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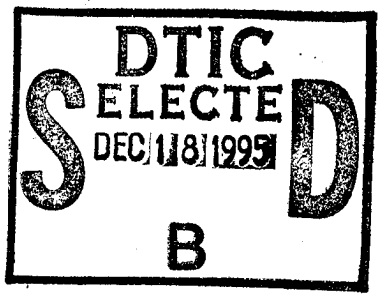
CONTRACT NO: DAMD17-92-C-2504



TITLE: Support for HIV-1 Intervention Therapy

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Rockville, Maryland 20850

REPORT DATE: October 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1995	Annual (30 Sep 94 - 29 Sep 95)

4. TITLE AND SUBTITLE
Support for HIV-1 Intervention Therapy

5. FUNDING NUMBERS
DAMD17-92-C-2504

6. AUTHOR(S)
Owen S. Weislow, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
SRA Technologies, Inc.
Rockville, Maryland 20850

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Since 1992 SRA Technologies, Inc. has provided services in support of DoD interventional trials for HIV. Three working groups were established in the areas of molecular biology, cellular phenotyping and drug testing. Production level services were provided for virus isolation, expansion, titration, sequencing, mutational analysis and drug susceptibility testing. Significant progress was made in the development of new assays for drug resistance, antibody neutralization, mutational analysis and for efficacy analysis of suggested by the discovery of factors from CD3/CD28 antibody stimulated cells that inhibit HIV Infection in Vitro.

14. SUBJECT TERMS
HIV, AIDS, Drug Testing, Anti-Virals, Neutralization, Gene Therapy, PCR, Mutational Analysis, Drug Resistance

15. NUMBER OF PAGES
71

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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★U.S.GPO:1991-0-299-935

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Owen S. Weislow 10/29/95
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INTRODUCTION

Contract DAMD17-92-C-2504, Support of HIV interventional trials, was initiated in October of 1992 and is administered by Dr. Owen S. Weislow of SRA Technologies, Inc.. This contract is intended to provide both research and development support of clinical studies in HIV antiviral chemotherapy, immunotherapy and immunoprophylaxis. The primary objectives of the contract, as stipulated in the contract award, are a) to develop assays to evaluate the viral and immune responses to anti-retroviral therapy including neutralization assays and drug susceptibility assays using clinical HIV isolates, b) to develop and validate assays that predict or demonstrate disease progression for use in interventional trials with an emphasis on molecular biologic approaches to viral characterization and quantification of viral burden, and c) to improve the accuracy, reliability, and cost effectiveness of clinical laboratory tests for all stages of HIV-1 and other retroviral infections. The contractor has, in consultation with the contract office's representative (Dr. Douglas L. Mayers, Capt, USN), incorporated three working groups to develop and optimize the necessary assays in support of these clinical studies and to establish production-level protocols. The three groups include the molecular biology working group, the cellular phenotype working group and the antiviral drug testing group. The work scopes of each are briefly described below with a detailed discussion of the progress made by each during the three base years of the contract. A Third section, the data group section, consisting of computer personnel from SRA, has been established to support the efforts of SRA to improve delivery of all necessary data elements to WRAIR personnel as stipulated in the contract award.

This is the final report describing progress made on contract DAMD17-92-C-2504 during the three base years from Oct. 1, 1992 thru Sept. 30, 1995. During this three year period a number of assays were developed or validated and moved into production status in support of ongoing clinical trials or as part of WRAIR's continuing efforts to improve patient management. Included in this arena are the molecular biology working group assays for the aa215 and aa74 mutations of reverse transcriptase (brought online in FY94), sequencing of RT for drug resistance and development of the capability to provide large-scale support for analysis of HIV viral burden. The latter capability is now being applied in support of WRAIR's HIV diagnostic and other collaborative efforts.

During the contracts three base years the cellular phenotyping group participated in the development and evaluation of a new infectivity reduction assay employed in virus neutralization studies and studied the neutralization kinetics of laboratory and field isolates of HIV with an eye toward improving existing assays. Particularly noteworthy are the studies performed by the cellular phenotype working group in support of WRAIR and the Naval Medical Research Institute's, Cellular Immunology Laboratory, evaluation of long term CD4+ cell cultures for *ex-vivo* use in investigations of genetic therapies. SRA's laboratories were instrumental in

demonstrating the resistance of CD4+ T-cells, stimulated with solid-phase, anchored CD3 and CD28 antibodies to HIV infection. We demonstrated this resistance to be active in nature, the result of soluble factors synthesized by CD4+ cells and derived from supernatants of these cells, that inhibit infection of cellular targets normally sensitive to HIV. Finally, the cellular phenotyping group also has scaled up studies of SI/NSI isolates, performed evaluations of antiviral gene construct and produced numerous large-scale expansions of viral stocks.

The antiviral drug testing group continues to evaluate clinical isolates for drug sensitivity as part of WRAIR's numerous collaborations and has, at the same time, evaluated many new putative antiviral compounds emanating from WRAIR's own laboratories and those of collaborators. Assays to accomodate preclinical evaluations of drug combinations were brought online and new, multiple drug resistant isolates have been identified, expanded, titrated and characterized as part of these efforts. Finally, the antiviral drug testing group has collaborated in the development of a new rapid screening system for drug resistance that could reduce the time to phenotype patients undergoing antiviral drug treatment. These activities are documented in the sections that follow below.

PROGRESS REPORT

1. Molecular Biology Working Group

During the first two years of this contract we improved and validated the PCR assay employed in testing for the presence of AZT-resistance associated mutations at HIV-1 reverse transcriptase amino acid's 215 position. In addition, we began application of this technology to a new clinical protocol (244) sponsored by the AIDS Clinical Trials Group (ACTG), NIAID and WRAIR. We have also begun efforts to develop a similar mutational assay for the 74 mutation that confers DDI resistance. Finally, we have made improvements in our diagnostic DNA sequencing protocols for use in support of drug-resistance monitoring in clinical trials and this past year brought on board, at SRA's expense, a new Perkin-Elmer 377 automated sequencer to accommodate any potential increase in WRAIR's sequencing requirements. Information on the rational behind these protocols and other background information is provided below, followed by copies of current protocols for each and a summary of work performed during the three contract years under review here.

Amplification Refractory Mutation System (ARMS)

Of great importance, in WRAIR clinical trials supported by SRA Technologies, is the monitoring of the acquisition of drug resistance. At this point in time, AZT (Zidovudine) is the primary drug used in the these trials. While potentially providing some benefit to the patient, the usefulness of AZT and other nucleoside

analogs as well as non-nucleoside RT inhibitors is limited by the tendency for almost all recipients to develop some level of resistance to the drug during the course of therapy. In order to better assess the effectiveness of treatment modalities, it is useful to have a rapid screening assay for patients that will indicate the onset of genotypic mutations associated with AZT resistance. During the course of this contract the 215 ARMS PCR assay was improved and applied to a nationwide clinical protocol for prosective analysis of the 215 mutation in clinical practice.

The theoretical basis for this assay was included in previous reports, but is described briefly here. A combination of ASO (Allele Specific Oligonucleotide) techniques and PCR has been developed that makes use of the best aspects of both techniques. Variously named the Amplification Mutation Refractory System (ARMS) or PCR Amplification of Specific Alleles (PASA), this technique takes advantage of the inability of synthetic oligonucleotide primers that are incompletely hybridized to a template to serve as effective PCR primers[1]. First described by Markham et al.[2] and Sommer et al.[3], this technique has been applied to the detection of single base changes and identification of specific alleles associated with disease in such diverse instances as cystic fibrosis[4,5], phenylketonuria[3], apolipoprotein genotyping[6] and HLA typing[7]. Larder et al. have applied this technique to examine AZT resistance acquired during chemotherapy, first, by characterizing the genetic mutations in the HIV RT gene that can be linked to *in vitro* resistance[8], and more recently by applying this technique to the direct determination of the presence or absence of these mutations in patient blood samples[9]. Further work by his group has validated and extended this approach [10-13].

In addition to the drugs currently available, a number of new agents are being developed and tested as HIV chemotherapeutic agents against both the HIV RT gene[14,15] and other viral targets such as the integrase protein[16], and the HIV protease[17]. It is expected that as the new agents and combination therapies are administered to patients, new mutations conferring resistance to these agents will also be discovered. It will be useful to monitor the appearance of resistant virus in patient populations in order to adjust the therapeutic regimes in use at the time and that is the intent of the ACTG's 244 protocol. Although the details of the assay described below are for the detection of the mutation at amino acid 215 that confers AZT resistance, this procedure is readily adaptable to the detection and monitoring of mutations at other locations within the viral RT gene simply by changing the primers used in the second (nested) PCR reaction and re-optimizing the PCR reaction conditions (if needed) to maximize sensitivity. Indeed, this has been accomplished at SRA for the 74 mutation associated with DDI resistance and our efforts in this regard are also documented elsewhere in this report. Our most recent incarnation of the 215 ARMS protocol for the detection of AZT resistant genotypes is described below.

ARMS Protocol

PCR Reactions - 215 Mutation Detection

We use A(35 mer) and NE1(35 mer) primers for the first set of cycles and the B and either 215M or 215W primers for the second set of cycles to detect mutant (resistant) or wild type (sensitive), respectively. These primers are identical to those described by Larder and Boucher (B. Larder, personal communication[18]). The primer sequences in use today are given below. The NE1(35), 215M and 215W are 5' biotinylated.

A(35)	TTGGTTGCACTTTAAATTTTCCCATTAGTCCTATT
NE1(35)	CCTACTAACTTCTGTATGTCATTGACAGTCCAGCT
B	GGATGGAAAGGATCACC
215M	ATGTTTTTTGTCTGGTGTGAA
215W	ATGTTTTTTGTCTGGTGTGGT

The PCR cycle part of the assay is identical whether the source material is plasma or tissue culture supernatants (viral RNA), or patient PBMCs or co-culture cells (proviral DNA). However, serum samples and samples treated with heparin have proven to be somewhat difficult to handle and 40 cycles has been the standard for the first cycle of amplification with those samples. Sample preparation steps are given for each substrate. New protocols for all steps of the ARMS assay are also provided.

Sample Preparation: PBMCs or Co-cultured Cells

1. Thaw frozen cells at 37°C and transfer to a sterile 15 ml polypropylene centrifuge tube.

2. Wash once with 10 ml PBS (2000 rpm 15 min.). Decant supernatant after wash and discard.

3. Add lysis buffer (10 mM Tris 8.3, 50mM KCl, 2.5mM $MgCl_2$, 0.45% NP-40, 0.45% Tween 20, proteinase K at 120 µg/ml) and resuspend pellet well for a cell concentration of 7.5 X 10^6 cells/ml. Be sure to lyse a negative control with the cell samples. Vortex briefly.

4. Incubate at 55°C - 60°C for 1 hr. Vortex before transfer in next step.

5. Transfer to 1.5 ml screw-cap microcentrifuge tube.

6. Heat-inactivate the proteinase K by incubating the tubes at 95°C for 15 min.

7. Transfer tubes to ice. Store lysates either at 4°C (no more than overnight) or at -20°C for longer periods.

Sample Preparation: Viral RNA from Plasma

Preparation of Plasma from Whole Blood

1. Centrifuge the whole blood at approximately 200 X g (1400 rpm) in a tabletop centrifuge at 4°C.

2. Remove the supernatant, taking care not to disturb the cell layer.
3. Centrifuge the supernatant from Step 2 at approximately 1000 X g (4000 rpm) in a tabletop centrifuge at 4°C. The purpose of this step is clarification, to remove residual cells.

4. If the plasma is to be extracted immediately, maintain on ice. If it is to be stored, aliquot into 1.0 ml cryo-vials, and store at -70°C.

Pelleting of Virus

1. If the sample is frozen, thaw quickly at 37oC, then maintain on ice.

2. Add 0.5 ml PBS/BSA to 1.5 ml screw cap tube. Tubes should be pre-labelled with specimen number.

3. Transfer 1 ml of plasma to each tube. Be sure to include negative control.

4. Pellet the virus by centrifugation at ≈12,000 x g (approximately maximum speed in a typical microcentrifuge at 4°C for 1h.

RNA Extraction

1. Remove and discard supernatant from the 1.5 ml tube by decanting and removing as much of the supernatatn as possible while the tube is inverted. Be careful not to disturb the pellet. Gentle tapping on clean gauze will help remove supernatant.

2. Add 800 µl Tri-Reagent (guanidinium/phenol). Vortex 15 s.

3. Allow to sit at least 5 min. at room temperature.

4. Add 160 µl $CHCl_3$ to each tube. Vortex 15 s.

5. Allow to sit at least 3 min. at room temperature.

6. Centrifuge at maximum speed (approximately 12,000 X g) in a microcentrifuge at 4°C for 15 min.

7. Remove the aqueous (upper, colorless) phase to a fresh tube.

8. Add 400 µl of cold isopropanol (IPA, 2-propanol) and 4 µl of 2.5 µg/µl tRNA to each tube. Mix well by vortexing.

9. Maintain at -20°C overnight.

10. Centrifuge at maximum speed in a microfuge at 4°C for 15 min.
11. Decant the supernatant.

12. Wash the pellet with 1 ml of ice-cold 75% ethanol. Vortex briefly.

13. Centrifuge at maximum speed (approximately 12,000 X g) in a microfuge at 4°C for 15 min.

14. Decant the supernatant.

15. Air dry the pellet. Do not use a Speed-Vac.

16. Add Virus Lysis Buffer A (1% NP-40, 0.04 mg/ml tRNA, 0.4 U/µl RNasin, 2 mM DTT) for 55 µl per 1 ml original specimen volume. Vortex.

17. Maintain at 42°C approximately 15 min. to fully dissolve the RNA, after which the extract should be stored at -70°C. The RNA should not be heated above 60°C until after reverse transcription, as RNasin in the Virus Lysis Buffer will be inactivated.

215 PCR Reaction Setup

A. DNA PCR:

The first set of PCR cycles uses A(35) & NE1(35) primers (NE1(35) primer is biotinylated) to produce a 805 bp fragment encompassing virtually all currently known drug resistance associated mutations in the HIV RT gene (amino acids 5-254 of RT).

PCR master mix:

7.1 µl H2O
22.4 µl dNTP (280 µM dNTP) (use dUTP only in the second PCR)
10.0 µl 10X 215 PCR buffer
10.0 µl Primers [A(35) & NE1(35); 250ng each.]

49.5 µl store at -20oC if required.
Add Taq polymerase (Promega, 5 units/µl) when ready to use.

When ready to begin PCR, aliquot 50µl of PCR mix with the Taq polymerase added to each reaction tube.

Add 70.0µl of oil overlay (may be omitted for PE 9600 cycler)
Add 50µl of sample lysate

NOTE: In contrast to the PCR protocol in use for HIV detection in our laboratory, dUTP and UNG are not used in the first PCR of this nested set. The use of dUTP significantly reduces the discriminating power of the 215W and 215M primers used in the second PCR reaction.

The 10X 215 PCR buffer contains:

500 mM Tris 8.3
250 mM KCl
15 mM $MgCl_2$
1 µg/ml BSA

1. In the positive control lab, add positive controls (10^4 sensitive and/or resistant cells) from freshly thawed stock dilutions to the appropriate PCR tubes.

2. Immediately carry the reactions to the cycler.

PCR Cycling Conditions for Perkin Elmer 9600 Cycler

First (Outer) PCR Reaction Cycling Conditions

1. 94oC 1' 15"
2. 94oC 30"
3. 55oC 30"
4. 72oC 2'
5. Repeat steps 2-4 for 18-30 cycles
6. Soak 72oC 10 min.
7. Soak 4oC

The precise number of cycles depends somewhat upon the expected number of infected cells. When testing co-cultured cells, 18 cycles is usually sufficient, due to the large number of infected cells in the population, whereas primary patient cells often require 30 cycles, while up to 40 cycles may be used to generate more product if needed for cloning and sequencing. Since there is no UNG in these reactions, they may be maintained at 25°C after last cycle until products are ready to be carried into second PCR. Yield of PCR products may be monitored by running a 10 µl aliquot of the first PCR reaction on an agarose gel. It should be noted that in cases of low numbers of virus, such as seen in patient PBMCs, no band may be visible after the first PCR. This is not necessarily an indication that the PCR failed however.

Reaction Setup for Second (Nested) PCR

The second (nested) PCR reaction utilizes the B and either 215W or 215M primers to discriminate between the wild type (AZT sensitive) or mutant (AZT resistant) genotype at amino acid 215 of the HIV-1 RT gene. The 215W primer recognizes either the Phe or Tyr mutant at amino acid 215 approximately equally.

1. Remove 10 µl of the **1st PCR reaction** and dilute 1:10 to 1:100 in water.

NOTE: The exact dilution can be varied to ensure clean discrimination between 215M and 215W primer products. In general, there is less than a 100X difference in the product yield between completely homologous primer/template combinations (sensitive virus DNA with 215W primer for example) and mis-matched primer/template combinations (sensitive virus DNA with 215M primer) (Dr. Frank White, unpublished observations). Because of this, if the quantity of product transferred into the second PCR (B & 215M/215W) is too high, cross-reactive bands appear in both sensitive and resistant reaction lanes. In this case, it is necessary to either dilute the products of the first PCR reaction further and repeat the second PCR, or repeat the first PCR with reduced cycle numbers. This latter approach generally gives slightly cleaner results, but is also more time and labor intensive. The second PCR is set up exactly as the first, with the addition of 10 µl of first reaction product and 90 µl of master mix containing the B and 215M or 215W primers. Note that dUTP can be substituted for dTTP in this reaction without affecting the specificity or sensitivity of the PCR. Uracil-N-glycosylase (UNG) may then be added to facilitate contamination control, as is the standard procedure for HIV detection PCR reactions.

PCR Master mix for second PCR (B & 215M/215W primers)

10.0 µl dNTPs (250 µM dNTP. May substitute dUTP for dTTP)
10.0 µl 10X 215 PCR buffer

10.0 µl primers (B & 215W, 215M) at the ratio of 1:2 total of B+215M or W should equal 250 ng/reaction
3.0 µl $MgCl_2$ (25 mM stock, 2.25 mM final concentration)

33.0 µl Store at -20°C until needed.

Add 56.3µl of H2O, 0.2 µl UNG (0.2 units, Epicentre Technologies) and 0.5 µl Taq polymerase (Promega, 5 units/µl) per reaction when ready to use.

When ready to begin PCR aliquot 90.0 µl total of the above mix to each reaction tube a 70.0 µl oil overlay (may be omitted for PE 9600 Cycler) and 10.0 µl diluted products from first PCR.

NOTE: There are slight changes in the concentrations of some of the reaction components (2.25 mM $MgCl_2$) between the first and second PCR reactions. These conditions have been optimized to increase product yield of the second PCR reaction.

Second (Nested) PCR Reaction Cycling Conditions

1. 25°C 3'
2. 94°C 5'
3. 94°C 1'
4. 48°C 30"
5. 72°C 30"
6. Repeat steps 2-4 for 30-40 cycles. Soak at 72°C after last cycle until products are either stored (-20°C) or analyzed.

B. For RNA PCR:

1. 215 RT PCR Master Mix:

DEPC H2O	3.2 µl
5X RT Buffer	10.2 µl
0.1 M DTT	2.0 µl
0.05 µg/µl NE1' primer	5.0 µl
10% NP-40	3.4 µl
25.0 mM dNTP's	1.0 µl
Total	24.6 µl/reaction

Store in -20°C freezer. Add 0.2 µl of RNasin (40 units/ml) and 0.2 µl of MMLV-RT per reaction when ready to use.

a. Add 25 µl of each RNA sample to a 96 well plate.

b. Overlay with 20 µl of mineral oil, seal and place in a heat block at 75°C for about 5 min. Immediately place on ice or at 4°C.

c. Add 25 µl of RT mix to each well.

d. Use either a 9600 cycler or manual transfer between heat blocks set for the following temperatures:

42°C 30 min.
99°C 5 min.

e. Place on ice or at 4oC for at least one min.

* Be sure that the tubes are pressed firmly in the heat block to ensure even heating.

2. A′ PCR master mix:

DEPC H2o	35.5 µl
10X "215" Buffer	5.0 µl
A′ primer (0.05 µg/µl)	5.0 µl
25 mM MgCl2	4.0 µl
Total	49.5 µl/rxn

Store Master mix at -20oC.
Add Taq Polymerase (2.5 units) in 0.5 µl/rxn to master mix before use.

When the RT reactions are completed, add 50 µl of A′ PCR mix to each tube and cycle as indicated in the 1st DNA PCR. Follow with 2nd PCR also as described for DNA PCR.

PCR Product Analysis

Figure 1 illustrates the sequences generated by the first PCR reaction and the second, nested PCR reaction. The second PCR product is 210 base pairs in length and is highlighted in bold letters and is bordered by the B primer or forward primer and the 215 discrimination primers (the wildtype in this example). This sequence was generated from HXB2RT. The aa215 codon is indicated by the enlarged lettering. Following the second PCR, 20 µl of the products are analyzed on a 3% agarose gel run in 1X TBE containing EtBr. Results are seen as bands in the lanes corresponding

to reactions containing either the B/215W (sensitive) or B/215M (resistant) primers. An example of a typical gel is shown in figure 2. A mixture of sensitive and resistant virus can be seen as bands in both lanes. However, this may also indicate that the second PCR reaction was overloaded with product from the first PCR. To eliminate this problem, the number of cycles for the first PCR can be reduced (from 30 to 15-22) and/or the dilution of products from the first PCR increased from 1:10 or 1:100 to 1:1000 or even 1:100,000 if needed to produce a single band from one or the other PCR reaction.

Application of the 215 PCR Assay to Clinical Samples

To date, over **700 patient PBMC samples** and more than **1000 patient plasma (RNA), serum or CSF samples** have been analyzed with 215 PCR assay as improved at SRA Technologies. SRA has been validated by the ACTG as a center for 215 mutational analysis by its participation in the QC validation program in support of WRAIR' ongoing collaboration with the ACTG. The ARMS assay is now being applied to the ACTG Clinical protocol number 244 to determine its usefulness in the management of AIDS patients on antiviral therapy. In addition to protocol 244, the ARMS assay for 215 has been applied to recent studies of plasma or serum viral RNA in seroconvertors (data not shown). These studies, performed for WRAIR in

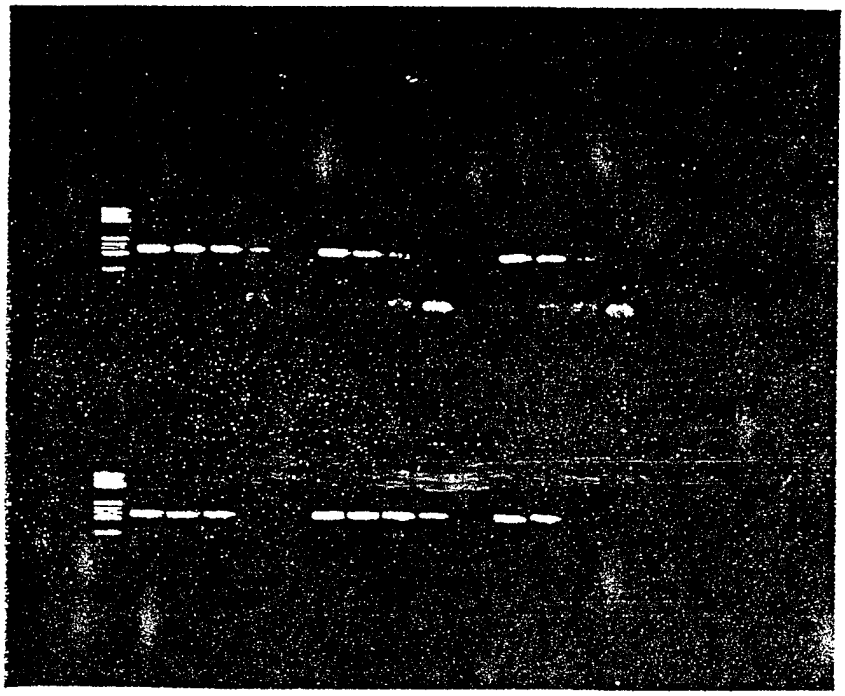
Figure 1

Products of the First and Second PCRs of the ARMS Assay for aa215 of RT

```
  1 CCC ATT AGC CCT ATT GAG ACT GTA CCA GTA AAA TTA AAG CCA GGA ATG
    GGG TAA TCG GGA TAA CTC TGA CAT GGT CAT TTT AAT TTC GGT CCT TAC

 49 GAT GGC CCA AAA GTT AAA CAA TGG CCA TTG ACA GAA GAA AAA ATA AAA
    CTA CCG GGT TTT CAA TTT GTT ACC GGT AAC TGT CTT CTT TTT TAT TTT

 97 GCA TTA GTA GAA ATT TGT ACA GAG ATG GAA AAG GAA GGG AAA ATT TCA
    CGT AAT CAT CTT TAA ACA TGT CTC TAC CTT TTC CTT CCC TTT TAA AGT

145 AAA ATT GGG CCT GAA AAT CCA TAC AAT ACT CCA GTA TTT GCC ATA AAG
    TTT TAA CCC GGA CTT TTA GGT ATG TTA TGA GGT CAT AAA CGG TAT TTC

193 AAA AAA GAC AGT ACT AAA TGG AGA AAA TTA GTA GAT TTC AGA GAA CTT
    TTT TTT CTG TCA TGA TTT ACC TCT TTT AAT CAT CTA AAG TCT CTT GAA

241 AAT AAG AGA ACT CAA GAC TTC TGG GAA GTT CAA TTA GGA ATA CCA CAT
    TTA TTC TCT TGA GTT CTG AAG ACC CTT CAA GTT AAT CCT TAT GGT GTA

289 CCC GCA GGG TTA AAA AAG AAA AAA TCA GTA ACA GTA CTG GAT GTG GGT
    GGG CGT CCC AAT TTT TTC TTT TTT AGT CAT TGT CAT GAC CTA CAC CCA

337 GAT GCA TAT TTT TCA GTT CCC TTA GAT GAA GAC TTC AGG AAG TAT ACT
    CTA CGT ATA AAA AGT CAA GGG AAT CTA CTT CTG AAG TCC TTC ATA TGA

385 GCA TTT ACC ATA CCT AGT ATA AAC AAT GAG ACA CCA GGG ATT AGA TAT
    CGT AAA TGG TAT GGA TCA TAT TTG TTA CTC TGT GGT CCC TAA TCT ATA

433 CAG TAC AAT GTG CTT CCA CAG GGA TGG AAA GGA TCA CCA GCA ATA TTC
    GTC ATG TTA CAC GAA GGT GTC CCT ACC TTT CCT AGT GGT CGT TAT AAG

481 CAA AGT AGC ATG ACA AAA ATC TTA GAG CCT TTT AGA AAA CAA AAT CCA
    GTT TCA TCG TAC TGT TTT TAG AAT CTC GGA AAA TCT TTT GTT TTA GGT

529 GAC ATA GTT ATC TAT CAA TAC ATG GAT GAT TTG TAT GTA GGA TCT GAC
    CTG TAT CAA TAG ATA GTT ATG TAC CTA CTA AAC ATA CAT CCT AGA CTG

577 TTA GAA ATA GGG CAG CAT AGA ACA AAA ATA GAG GAG CTG AGA CAA CAT
    AAT CTT TAT CCC GTC GTA TCT TGT TTT TAT CTC CTC GAC TCT GTT GTA

625 CTG TTG AGG TGG GGA CTT ACC ACA CCA GAC AAA AAA CAT CAG AAA
    GAC AAC TCC ACC CCT GAA TGG TGT GGT CTG TTT TTT GTA GTC TTT

670 GAA CCT CCA TTC CTT TGG ATG GGT TAT GAA CTC CAT CCT GAT AAA TGG
    CTT GGA GGT AAG GAA ACC TAC CCA ATA CTT GAG GTA GGA CTA TTT ACC

718 ACA GTA CAG CCT ATA GTG CTG CCA GAA AAA GAC
    TGT CAT GTC GGA TAT CAC GAC GGT CTT TTT CTG
```

Figure 2

Typical Gel from ARMS Assay for Genotypic Resistance or Sensitivity to AZT



Lane #	Description	Result
1	Molecular Markers	
2	Specimen A 1:1000 Dilution	
3	Specimen A 1:10000 Dilution	
4	Specimen A 1:100000 Dilution	Sensitive
5	Specimen A 1:1000000 Dilution	
6	Space	
7	Specimen B 1:1000 Dilution	
8	Specimen B 1:10000 Dilution	
9	Specimen B 1:100000 Dilution	Resistant
10	Specimen B 1:1000000 Dilution	
11	Space	
12	Specimen C 1:1000 Dilution	
13	Specimen C 1:10000 Dilution	
14	Specimen C 1:100000 Dilution	Mixture
15	Specimen C 1:1000000 Dilution	

collaboration with laboratories in Swizterland, provided some of the earlist evidence for the transmission of AZT resistant HIV-1 (see publication and abstract citations in the Appendix to this report). SRA has also performed more than **180 assays for DDI mutations at codon 74 in the RT gene.**

Additional ARMS Assay Development

One major limitation of the currently used agarose gel based assay is that it does not allow accurate evaluation of patient samples containing mixtures of resistant and sensitive virus. Theoretically, the presence of a mixture of resistant and sensitive virus in a patient sample would produce PCR products from both the resistant and sensitive primer sets. These would be seen as bands appearing in both sets of lanes on a gel. Due to incomplete inhibition of primer extension from mismatched primers (such as the 215W primer hybridized to a resistant virus) however, it is possible to produce diagnostic bands of the gel from both the sensitive and resistant PCR reaction from samples that contain only one species of virus by simply overloading the second PCR reaction with product from the first (A & NE1 primer set) PCR reaction. Efforts to improve on this rather subjective procedure for analysis have not been successful. They included attempts to quantitate products of the second PCR using biotinylated and fluorescently tagged primers. Readout was on Molecular Dynamics FluorImager™ and both gel and capture plate formats were studied. Though product differentiation was possible, quantitation proved problematic and efforts in this direction were curtailed.

Currently we're pursuing additional modifications to the 215 protocol with the intention of increasing the sensitivity of the assay when analyzing plasma RNA samples, and to improve the quantitative ability of the assay. A new assay procedure, available through Perkin-Elmer, the TaqManTM PCR procedure may prove more fruitful and studies, in collaboration with Perkin-Elmer's applications group have been ongoing. SRA (using its own funds) has also begun a series of independent investigations we hope leads to quantitative mutation assays with general applicability. Both nucleotide addition and fluorescently tagged primer assays are being evaluated using the 377 automated sequencer and GeneScan software from Perkin-Elmer.

Diagnostic DNA Sequencing

Despite the effectiveness of the 215 PCR protocol described in the previous section, it is limited in that it can only be used where the site of mutation is known, and then only when the surrounding sequence is conserved sufficiently to ensure efficient primer hybridization. For new drugs, where the site of the resistance-conferring mutation is not well characterized, or for mutations occurring in hypervariable regions, diagnostic sequencing is the only method that can provide useful genotypic

information. During the first two years of the contract a manual sequencing procedure was developed, underwent a number of modifications and was subsequently supplanted by an automated procedure that employs an ABI 377 sequencer based on the use of fluorescence. However, the bulk of sequencing requests recieved on behalf of WRAIR were carried out using the manual approach, a protocol for which is supplied below.

Our latest procedure for sequencing of a portion HIV's RT gene is as follows:

Sequencing Step-by-Step Procedure

A. Template Preparation

It is recommended that PCR products be generated by a method called "Ampliwax Hot Start". Better sequencing results have been obtained using "Hot Start" PCR products because fewer non-specific products are generated.

1. Purify template by placing into the retentate cup of a Microcon 100 with 300 µl TE buffer.

2. Microcentrifuge at 3,000 RPM's for 9 minutes.

3. Empty the waste in the centrifuge tube and repeat step #2 two more times, each time washing with 400 µl of TE buffer.

4. After the last wash, remove the cap and place retentate cup into a clean catch tube and turn upside down, so that the sample reservoir is inside the centrifuge tube. Centrifuge at 1,000 RPM's for 5 min.

B. Sequencing Reaction

1. Label four 0.5 ml centrifuge tubes A,G,C and T. Place 3 µl of ddATP termination mix into the tube labeled A, and do the same for G,C and T tubes. Store on ice.

2. In a separate 0.5 ml centrifuge tube, combine the following:

1/20 volume of purified PCR product
2 pmole Primer
4 µl 10x sequencing buffer
1 µl a ^{33}P dATP (10 µCi)
1 µl Exo- PFU polymerase (2.5 U)
Bring volume to 26 µl with ultra pure water
4 µl DMSO

---

final volume = 30 µl

Mix well by pipetting and a brief spin in the centrifuge. Keep on ice.

3. Immediately aliquot 7 µl of the reaction mixture from step #2 into each of the 4 termination tubes containing 3 µl of their appropriate ddNTP. Mix thoroughly, making sure reaction mix and dideoxynucleotide mix are at the bottom of the tube.

4. Overlay the reaction with 15 µl mineral oil. Briefly centrifuge.

5. Cycle reactions as follows:

   Denature at 95°C for 5 minutes
   95°C for 30 s
   60°C for 30 s 30 cycles
   72°C for 1 min.
   Hold at 72°C

   ***Note:** Reactions should not be held for more than 5 minutes at 72°C, degradation can occur. The cycling takes approximately 1 1/2 - 2 hours.

6. At the end of the cycling procedure, add 5 µl of stop solution to each reaction tube and immediately place on ice.

7. When ready to load gel, denature samples at 75-80°C for 5 minutes, place on ice and load 3 µl on gel. Otherwise, samples can be stored at -70°C until ready for use.

Sequence Data Analysis

Currently, manual DNA sequence information is acquired with a Howtek Scanmaster 3+ flat bed scanner used to digitalize the entire 35X43 cm film of the sequencing gel. Using input data from digitized films the Millipore/BioImage analysis software SRA currently runs on a Sun Sparc 10 workstation can automatically define the sequencing reaction lanes and perform automatic base calling using neural network algorithms for increased accuracy. These advanced algorithms can be "trained" to improve base-calling accuracy the more they are used. In addition, the software allows quantitation of band intensity from the sequencing gel, facilitating determination of mixed population at a particular base location based in the sequencing gel information.

Table 1

Sample	Amino Acid # 18	40	62	98	100	108	136	150	160	180	181	192	205	214	229
HIVHXB2	GGC (Gly)	GAG (Glu)	GCC (Ala)	GCA (Ala)	TTA (Leu)	GTA (Val)	AAC (Asn)	CCA (Pro)	TTC (Phe)		TAT (Tyr)	GAC (Asp)	CTG (Leu)	CTT (Leu)	TGG (Trp)
DRUG M RESIST.			GCC (Ala) GTC (Val)	GGA (Gly) GCA (Ala)		ATA (Ile)	AAT (Asn)	-						CTT (Leu) TTT (Phe)	
DRUG N RESIST.		GAA (Glu)			ATA (Ile)								CTA (Leu)		
DRUG O RESIST.						ATA (Ile)			TTT (Phe)					TTT (Phe) CTT (Leu)	TGG (Trp) TTG (Leu)
DRUG P RESIST.	GGC (Gly) GGT (Gly)										TGT (Cys)				
DRUG Q RESIST.		GAA (Glu) GAG (Glu)						-			TGT (Cys)	GAC (Asp) GAT (Asp)			
IIIB							AAC (Asn) AAT (Asn)	-							

This system is currently connected to our LAN (described in the reports section) and can access other computers both in-house and through the Internet. The system can also interact with other molecular biology software packages, such as the DNAStar Lasergene system currently running on a Mac IIci with Genbank on CD-Rom. Custom filters have been provided to directly access the Mac format DNAStar files for use on the SUN system. These capabilities will provide easy access to the sequence data generated in our laboratories, and the information generated can easily be included in requested reports.

During the three base contract years SRA's manual sequencing protocols underwent a number of procedural modifications intended to reduce error rates frequently associated with the use of Taq DNA polymerase. When using PCR based sequencing techniques, several potential problems may arise. Several studies have examined the error rate of *Taq* DNA polymerase when used in a PCR assay[19-21]. SRA now employs the recently introduced thermostable DNA polymerase (*Pfu* DNA polymerase, Stratagene Inc.), which is possessed of a 3'-exonuclease proofreading activity, that significantly reduces the chance of PCR induced errors in sequence determination[22]. *Pfu* polymerase has now been substituted for NEB CircumVent™ in all DNA manual sequencing reactions at SRA.

During the past year, SRA performed sequencing in support of WRAIR's collaborations with the ACTG. AT the COR's request we have become active members of the ACTG's virology committee's sequencing "Swat team, " and have sequenced a number of QC panels in support of this effort. Halfway through the fiscal year, SRA Technologies acquired a Perkin-Elmer ABI Prism 377 automated DNA sequencer, making us one of the first sites to obtain the new model. The most recent of the ACTG QC samples were sequenced by both manual and automated methods, yielding nearly identical results. SRA's automated sequencing capabilities rapid throughput of DNA sequencing samples and larger batch sizes. During the three base years of the contract SRA staff manually **sequenced the RT gene from more than 41 Isolates or cell pellets representing more than 30 Kilobases** and have been **validated as an ACTG sequencing site** by virtue of our participation in QC validations on WRAIR's behalf. Table 1 presents an example of a manual sequencing run. These data were originally presented in the FY93 annual progress report. All future efforts will utilize the more efficient automated approach.

Viral Burden Assays

SRA began development of a quantititative RT-PCR at the start of the first contract year. This assay, an avidin-biotin capture plate-based procedure, required extensive evaluation and validation and proved, until recently, too variable and inconsistent to be employed for any serious evaluations of viral burden. The assay has since been modified and is available to serve as a backup to the widely utilized Roche Amplicor

kit and the more loborious, but exceedingly sensitive procedure developed by Dr. Vahey of the Henry Jackson Foundation. At the beginning of the last contract year, it was decided that WRAIR would provide SRA Technologies with an older model a phosphorimager (donated by Dr. Vahey). The intention was to encourage transfer of this technology to SRA where a production level procedure might be put in place to satisfy WRAIR's requirements for large-scale viral burden analyses. Previously, we had been supporting Dr. Vahey's work by preparing viral RNA from patient plasma, which was then sent to the Jackson Foundation for further analysis. During November and December of 1994 (FY95), two members of the SRA staff were trained in the assay, and the phosphorimager was transferred to SRA in late December. It took SRA a while to get the assay up and running, for several reasons. The phosphorimager was damaged while it was in shipment to SRA, and it took several weeks for it to be repaired. Second, the RNA transcript that was used for the

Amplicor HIV Monitor Calculations

Control ID: 14887

Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS Copies	Sample DF	HIV Copies/ ml
760.950160	.441	x 125	55.125	15.841	x66	x40	41819
	.696	x 5	3.48				
	QS OD	QS DF	OD*DF				
760.950113	-.029	x 25	ND		x	x40	ND
	.789	x 5	3.945				
	QS OD	QS DF	OD*DF				
760.VB001	.482	x 625	301.25	91.985	x66	x40	242840
	.655	x 5	3.275				
	QS OD	QS DF	OD*DF				
760.VB002	.916	x 25	22.9	10.701	x66	x40	28250
	.428	x 5	2.14				
	QS OD	QS DF	OD*DF				
760.VB003	.484	x 25	12.1	3.380	x66	x40	8923
	.716	x 5	3.58				
	QS OD	QS DF	OD*DF				
760.VB014	-.029	x 625	ND		x	x40	ND
	-.023	x 5	ND				
	QS OD	QS DF	OD*DF				
760.VB017	.451	x 25	11.275	5.113	x66	x40	13499
	.441	x 5	2.205				
	QS OD	QS DF	OD*DF				
760.VB001	.491	x 625	306.875	77.887	x66	x40	205622
	.788	x 5	3.94				
	QS OD	QS DF	OD*DF				
760.VB002	.267	x 125	33.375	9.646	x66	x40	25465
	.692	x 5	3.46				
	QS OD	QS DF	OD*DF				
760.VB003	.433	x 25	10.825	2.887	x66	x40	7621
	.75	x 5	3.75				
	QS OD	QS DF	OD*DF				
760.VB014	-.028	x 625	ND		x	x40	ND
	-.021	x 5	ND				
	QS OD	QS DF	OD*DF				
760.VB017	.679	x 25	16.975	5.330	x66	x40	14070
	.637	x 5	3.185				
	QS OD	QS DF	OD*DF				

For Research Use Only.

Table 2

standard curve did not work, and had to be prepared a second time. Finally, a contamination problem was discovered that previously had been difficult to detect. This contamination problem was probably caused, in part, by the large volume of PCR experiments done at SRA. It appears that this contamination stemed from our use of UNG in most PCR reactions. Since UNG is not used in the Viral Burden Assay, it is more sensitive to carryover contamination.

We assayed over 50 samples for viral burden utilizing the Liquid Hybridization procedure. In addition, **we have extracted well over 1100 samples for viral burden analysis** by Dr. Vahey's laboratory during the period covered by this report.

Recently, it was decided most viral burden studies would be accomplished using the Roche Amplicor HIV-1 quantitative PCR assay. The liquid hybridization protocol will no longer be supported by this contract and the equipment loaned to us has since been returned. We currently have two laboratory scientists trained and certified by Roche in use of the Amplicor viral burden assay. We are in the process of **analyzing the viral burdens of 250 heparinized CPCRA samples** using the Amplicor procedure unfortunately supplemented by a rather laborious extraction procedure employing silica. The heparinized plasmas, available to the CPCRA has necessitated this approach and the project will not be completed until sometime in early Novermber as a result.

In support of this effort, **SRA has developed a computer program**, utilizing Oracle and its laboratory information management system t**hat automates parts of the viral burden analysis**. A sample of this software's output is shown in Table 2 above.

2. Cellular Phenotype Working Group

The Cellular Phenotype Working Group worked in three major areas during the three base contract years. These included; 1) developing and validating efficent phenotypic assays to support evaluation of neutralizing antibodies in vaccinees, 2) providing newly expanded and titrated stocks of primary HIV-1 isolates for WRAIR investigators along with phenotypic characterizations of isolate cytopathicity in support of various WRAIR protocols and 3) developing and implementing *in vitro* systems to evaluate antiviral genes for the treatment of HIV disease. The progress made during the contract period in each of these areas is reviewed below.

Development and Optimization of Virus Titration and Neutralization Assays

The majority of virus neutralization studies reviewed here were completed during the contact's first two years. No neutralization work was performed in the last of the contract's three base years. Virus neutralization assays may be employed to identify and differentiate virus, as well as to determine the host immune responsiveness to

a specific viral infection and/or vaccination with various viral protein(s). Although identification of serum antibodies which inhibit viral infection *in vitro* may be a useful marker of protective immunity for some viruses *in vivo*[23], the significance of neutralizing antibodies in influencing clinical outcome in HIV infected individuals is not well understood.[24-30] Currently, there are a number of assays being used to evaluate the effect of antibody on HIV replication. Originally, most studies utilized immortalized cells (*e.g.*, H-9, MT-2, etc) that exhibited susceptibility to one or more laboratory strains of HIV (IIIb, RF, MN, etc). Susceptibility was usually evaluated by the production of viral markers and/or the induction of cytopathic effects (CPE).[31-35] However, most field isolates of HIV (*i.e.*, low passaged, patient isolates) infect immortalized cells with very low efficiency, thus these assays are of limited value in assessing the neutralizing antibody titers of a patients sera to clinical isolates.

During the first contract year considerable effort was placed in defining the conditions for optimum growth of HIV in PBMCs and a number of particularly senstive leukopacks were chosen for subsequent use in all studies. We developed procedures for the evaluation of leukopack-derived donor PBMCs for sensitivity to HIV infection. This was important for two reasons. First, the asssays we were required to develop were short term systems usually of four days duration. This necessitated the production of measureable levels of HIV p24 (the endpoint for the majority of studies) in a short span of time. Secondly, many of the clinical isolates employed in this work were slow growers, producing only low levels of p24 over the required four day period of culture. Thus use of only the most virus sensitive PBMCs was required. Using a pre-neutralization virus titration procedure, developed during the past year, we analyzed a number of donor leukopack-derived PBMCs for sensitivity to virus infection by slow/low (from early stage patients) and rapid/high variants of HIV (from late stage AIDS patients) on donor cells. The best donors, i.e. those that provide the highest titers in the shortest period of time are selected, in some cases pooled, and whenever possible, used throughout the entire process of virus titration, characterization and neutralization (or other immunoassay analyses) as required.

The data in Table 3 illustrate SRA's use of the aforementioned pre-neutralization titration protocol for evaluation of cyropreserved PBMCs obtained from eight normal donors. The cells were tested in a single experiment against four separate isolates of HIV-1. These isolates were donated by investigators at the Walter Reed Army Institute for Research (WRAIR) and the Henry M. Jackson Foundation (HMJF) who classified them as rapid/high (9881 and 873b) or slow/low (8871 and 4026).

Table 3

EFFECT OF DONOR PBMCs ON VIRUS TITER

PBMC DONOR	VIRUS ISOLATE 4026	8736	8871	9881
13108	1024*	2048	12944	131072
14486	NT	512	648	10369
14557	2048	1024	12944	>131072
14659	8192	3236	65536	>131072
16952	20738	1024	4096	>131072
17679	128	2048	676	32768
18072	809	648	12944	103552
18251	1024	512	5404	51776

* $TCID_{50}$ determined in a pre-neutralization virus titration assay and calculated by the method of Spearman-Karber.

The data suggest that a remarkable variability exists between donor PBMCs as regards their ability to support replication of these isolates. The reasons for this variability are not immediately evident.

As noted above, it is SRA's intention to utilize single leukopack donor-derived PBMCs for all phases of neutralization study including virus titration and neutralization. To ensure that sufficient numbers of PBMCs are available for *in vitro* studies we have evaluated a single donor's multiple bleeds, individually and as cell pools, for sensitivity to infection with rapid/hi (9881) and slow/low (4971) variants of HIV. The data generated in this study are summarized in Table 4 below. In one experiment leukopacks 20841, 21480 and 18251 were tested individually for sensitivity to virus in a pre-neutralization titration. Leukopacks 20841 and 21480 were clearly more sensitive to virus replication at days 4 and 7 when compared to 18251.

Table 4

EFFECT OF LEUKOPACK POOLS ON VIRUS TITERS

	Leukopack Pools				
		Exp 1		Exp 2	
Virus Isolate	20841	21480	18251	20481 21480	18251 20481 21480
9881					
Day 4	5,404*	6,562	2,048	26,249	4,096
Day 7	65,536	32,768	8,192	419,991	14,263
4971					
Day 4	NT	NT	NT	2,048	2,048
Day 7	NT	NT	NT	14,263	5,404

* $TCID_{50}$ determined in a pre-neutralization virus titration assay and calculated by the method of Spearman-Karber.

A second, independent, experiment was performed using pools of these 3 leukopacks to determine the influence of a poorly performing leukopack on a pool of the more sensitive cells. These data show that the addition of 18251, a less sensitive bleed, to a pool of 20481 and 21480 (better individual performers) significantly reduced the titers obtained at days 4 and 7 for the 9881 isolate. The mechanism responsible for this effect is not immediately clear, but initial observations suggest that adherent cell populations appearing on flask surfaces during PHA stimulation of 20481 and 21480 are markedly reduced during stimulation of 18251 and the 3 leukopack pool. These data again point to the importance of pre-screening all donor PBMCs prior to their use in neutralization assays.

Earlier studies in our laboratory have suggested that fewer numbers of cells per well at the start of culture result in better cell growth and higher titers of virus produced. The results in Table 5 summarize those initial studies and demonstrate that for rapid/hi isolates in particular

Table 5

Effect of Cell Density on Cell Proliferation and Virus $TCID_{50}$

Num of Cells/well		Fold Increase in Cell Numbers		$TCID_{50}$	
	Virus	Day 4	Day7	Day 4	Day 7
5×10^4					
	4971.5	8.8	12.8	891	1024
	9881			7131	10809
2 x 105	4971.5	2.9	2.9	675	445
	9881			2048	2048

* $TCID_{50}$ determined in a pre-neutralization virus titration assay and calculated by the method of Spearman-Karber.

(9881 is but one example) fewer numbers of cells per well may result in significantly improved cell growth and higher early titers of virus. Indeed, prior to using any leukopack or pool of leukopacks these types of analyses will be performed to maximize use of available materials. During the past contract year the preneutralization procedure was used to analyze some 25 individual and pooled leukopacks for virus sensetivity against slow/low and rapid/high isolates. Determinations of virus titers were made for no less than 18 international viral expansions and for at least 40 viruses in multiple leukopacks as a prelude to various experimental procedures. This work represented more than 8400 individual cultures of PBMCs.

Several neutralization projects were completed during the first two base years of the contract; these fall into two categories:

1. Testing of vaccinee sera to determine neutralizing potential and specificity, if any, of candidate vaccines for a panel of laboratory virus strains and selected virus isolates, and

2. Screening and titration of naturally-occuring (infected patients) immune sera against viral isolates in an attempt to determine if previously determined genotypes correlate with apparent serotypes.

The general strategy for both categories has been to screen sera against a typical laboratory strain and several clinical isolates; then to carefully characterize the

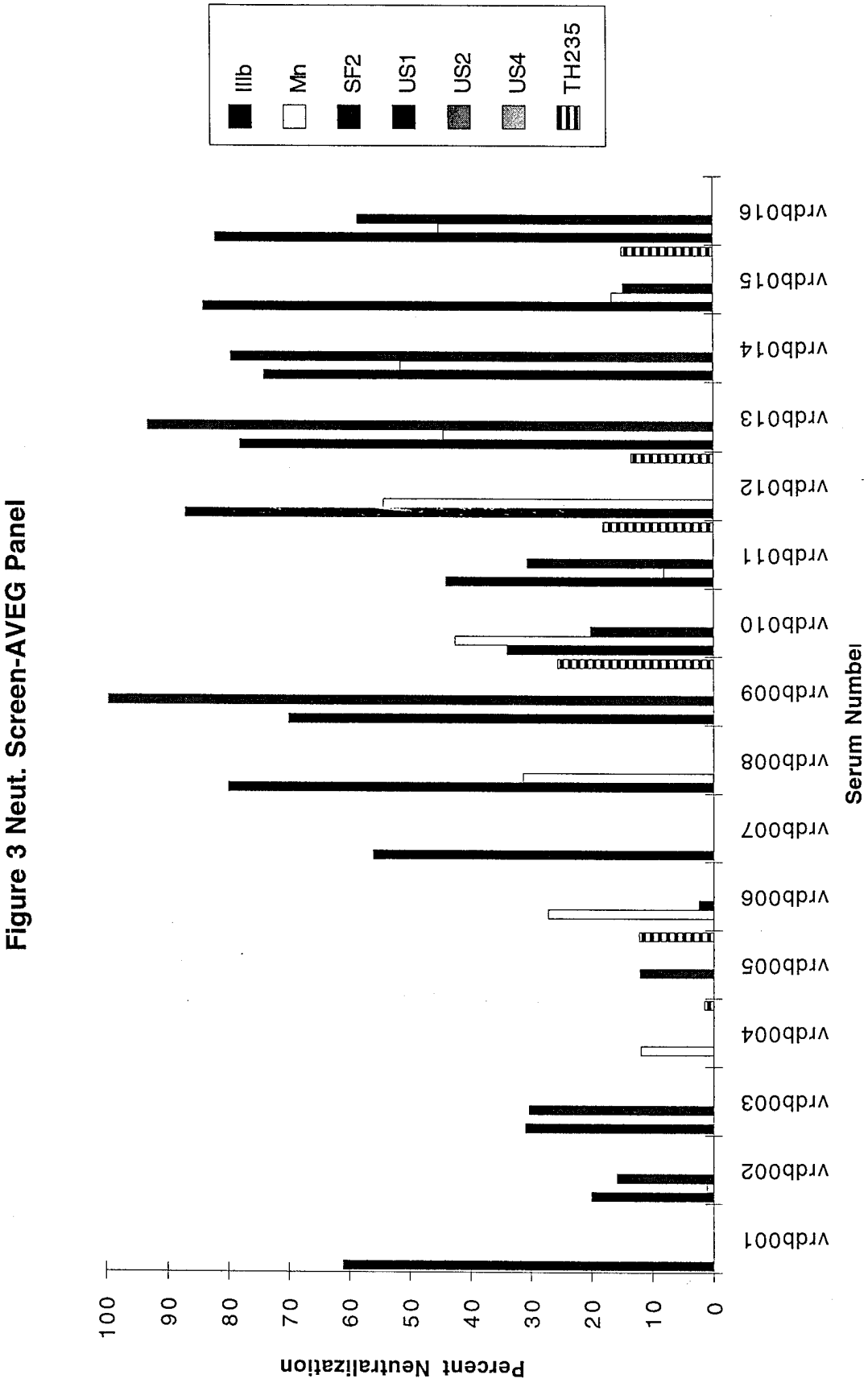


Figure 3 Neut. Screen-AVEG Panel

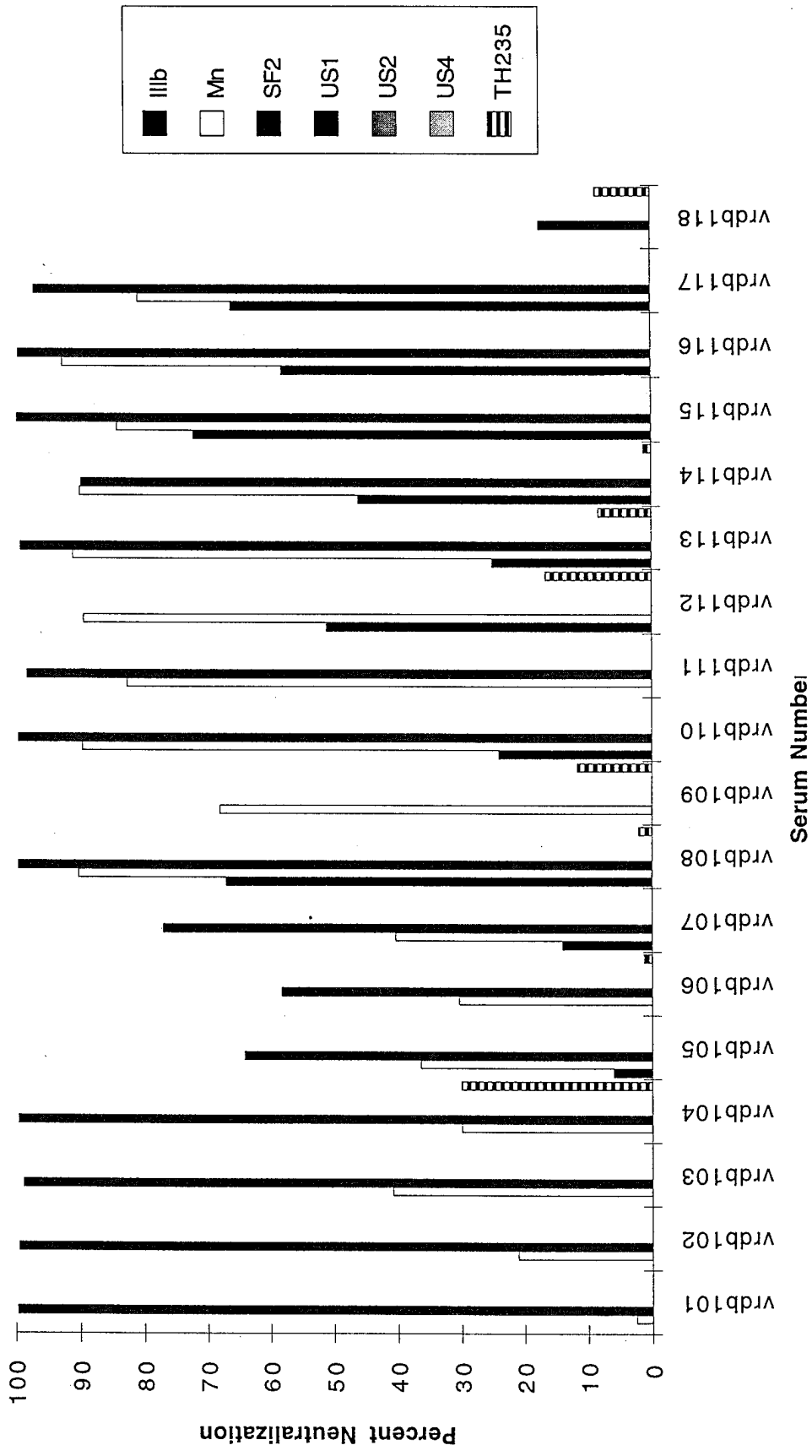


Figure 4 Neut. Screen-AVEG Panel

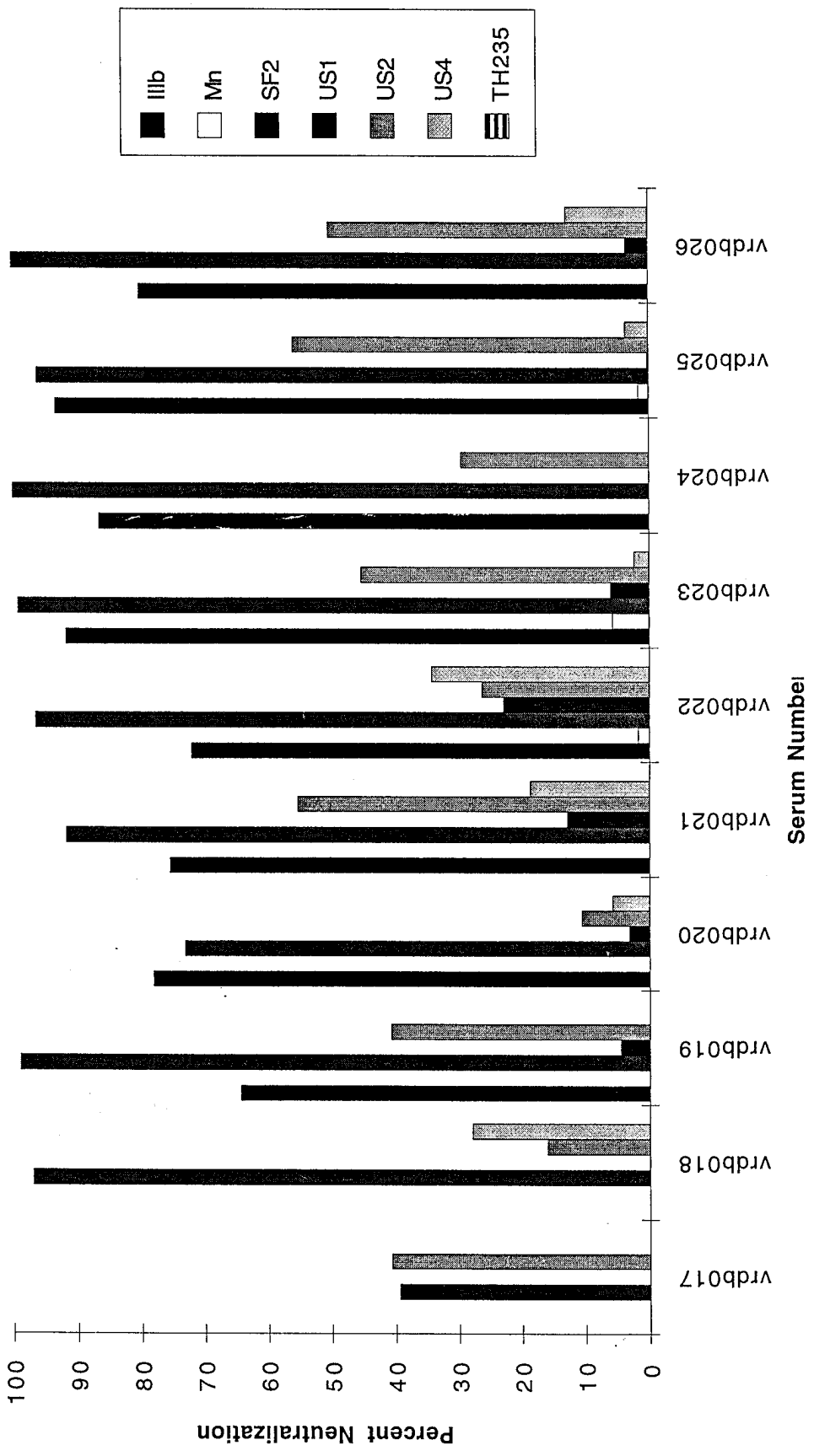


Figure 5 Neut. Screening-AVEG Panel

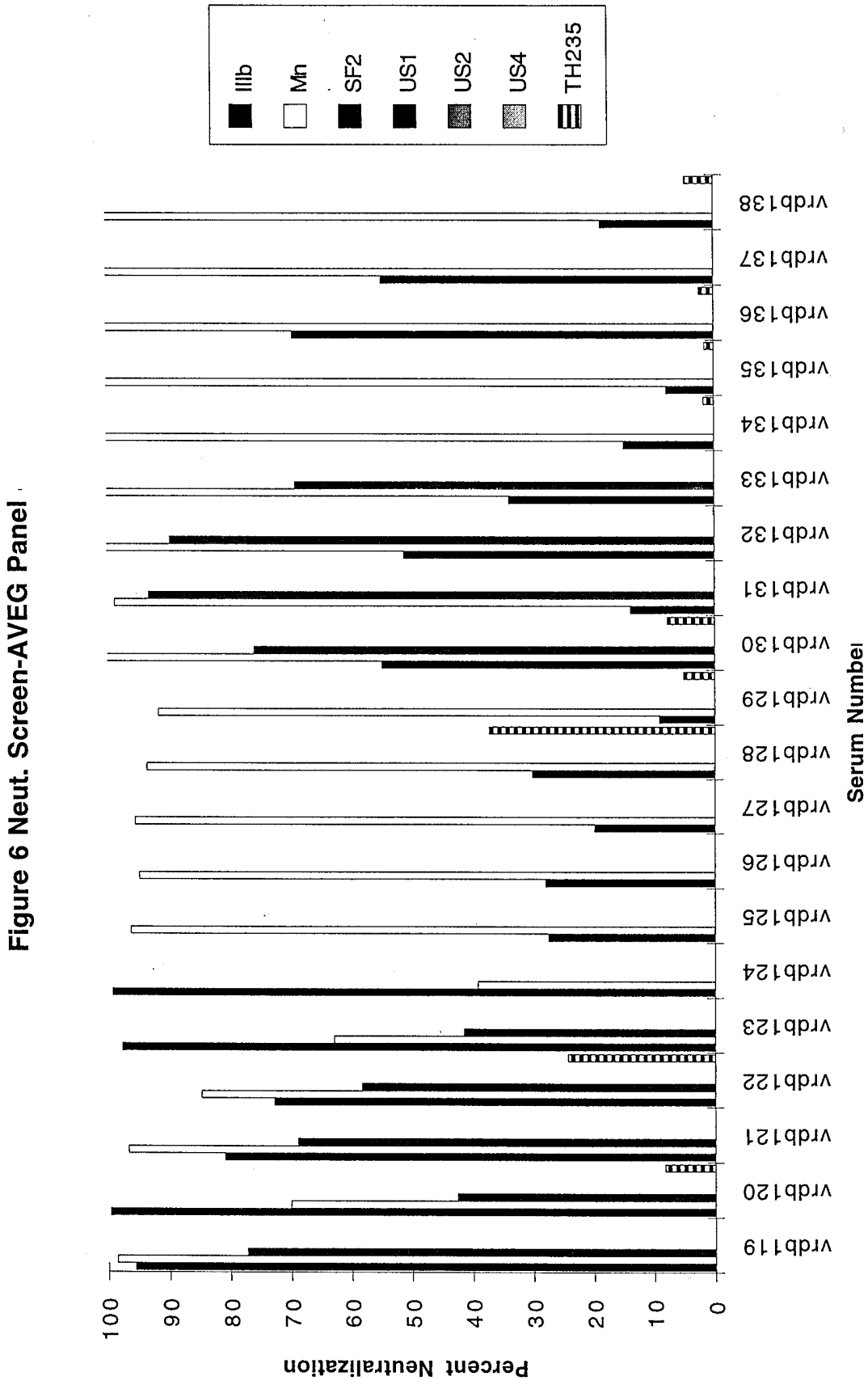


Figure 6 Neut. Screen-AVEG Panel

Figure 7 Neut. Screen-lab Strains & Primary Isolat

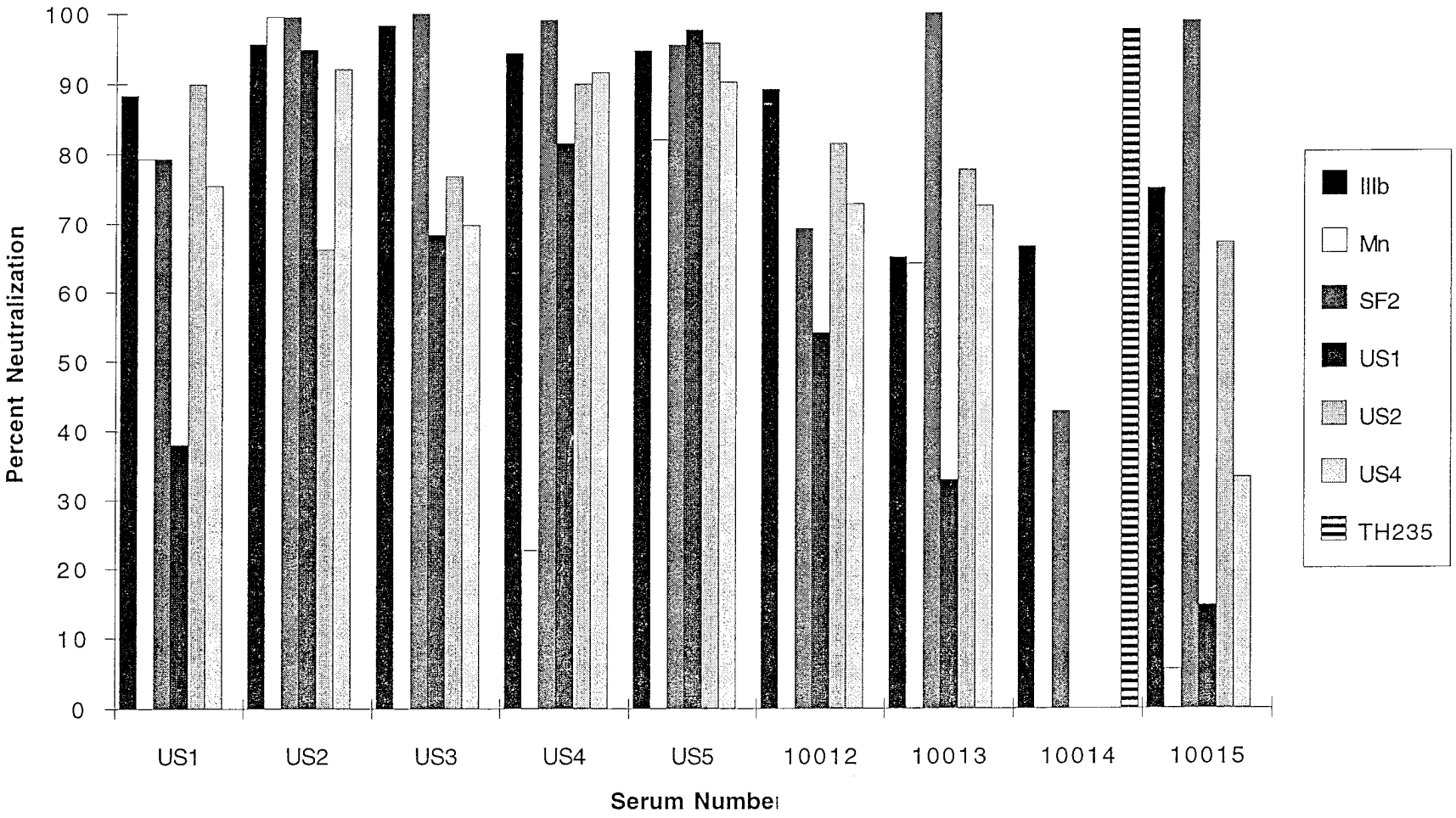


Table 6 Neutralization of Lab Strains of HIV By Patient Sera

	Mn		SF2		IIIb	
SERUM ID	% Neut	Titer	% Neut	Titer	% Neut	Titer
US3	99	14000	99	3500	94	1400
US5	99	26000	99	6200	96	210
Seroneg	<50	<10	<50	<10	<50	<10
VRDB008	<50	<10	<50	<10	86	90
VRDB018	94	110	<50	<10	<50	NT
VRDB103	80	70	90	150	<50	<10
VRDB117	95	12700	96	1500	78	50
VRDB118	<50	<10	<50	<10	<50	<10

* NT: Not Titered

Figure 8 Neut. Screen-International Isolat

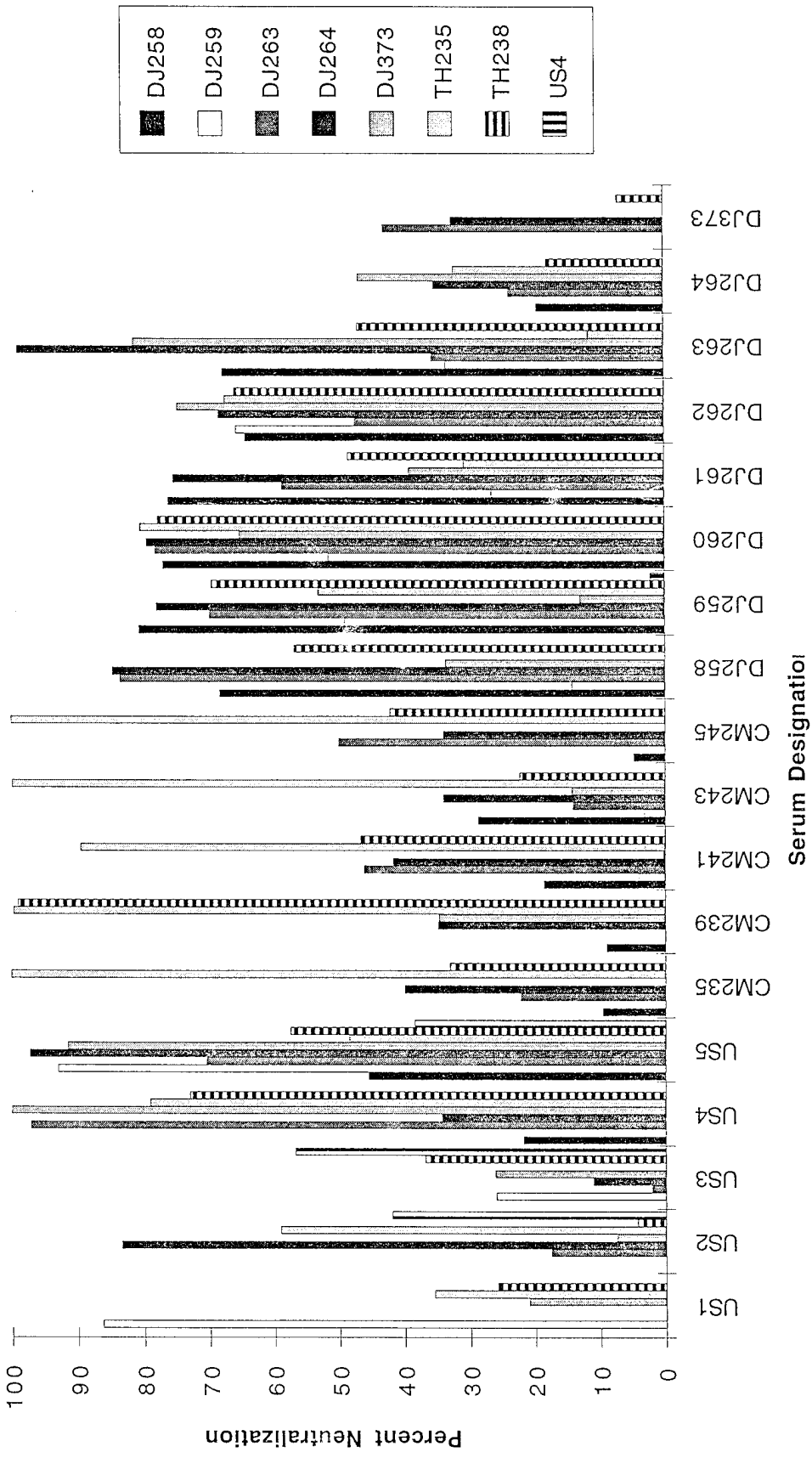


Table 7 SUMMARY OF WHO SERUM SCREENING RESULTS

VIRUS

	Group A.				Group B				Group C		Group D		Group E	
	RW09		UG37		BR20		TH14		BR25		UG24		TH22	
SERUM	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index
RW09	26088	1.50	5418	1.25	24447	2.22	7235	3.19	2858	3.16	318000	2.66	3170	2.77
UG37	18581	2.11	3038	2.24	8408	6.46	3385	6.83	167	53.98	148914	5.68	1034	8.51
BR20	11813	3.31	5465	1.24	20743	2.62	5932	3.90	3885	2.32	432200	1.96	2758	3.19
TH14	21867	1.79	5281	1.29	27202	2.00	7155	3.23	3095	2.92	229200	3.69	4301	2.05
BR25	25625	1.53	4857	1.40	26348	2.06	6269	3.69	3392	2.66	388800	2.18	3601	2.44
TH22	14074	2.78	6134	1.11	25680	2.12	11859	1.95	4856	1.86	200781	4.21	3129	2.81
UG24	23074	1.70	3872	1.75	18243	2.98	3347	6.90	1826	4.94	101886	8.31	1641	5.36
FDA2	9892	3.96	127	53.55	627	86.59	418	55.28	347	25.99	356400	2.37	1033	8.52
NHS	39130	1.00	6790	1.00	54324	1.00	23106	1.00	9028	1.00	846200	1.00	8797	1.00

Table 8 SUMMARY OF WHO VIRUS REDUCTION ASSAY

TCID50 (STANDARD DEVIATION) IN THE PRESENCE OF:

	NHS(1)		NHS(2)		NHS (mean)		10013		9170	
VIRUS	TCID50	STDEV	TCID50	STDEV	TCID50	STDEV	TCID50	STDEV	TCID50	STDEV
TH235	127	27	143	9	135	6	29	3	96	6
US1	1460	43	510	21	808	22	1490	44	33	3

log[TCID50(NHS)/TCID50(test)]

	NHS				
VIRUS	NHS(1)	NHS(2)	(mean)	TH243	US2
TH235	0.03	-0.03	0.00	0.67	0.15
US1	-0.26	0.20	0.00	-1.04	0.62

AVEG can be seen in figures 3-7. The data presented indicate that sera from vaccinees--immunized with glycoproteins from laboratory strains--are able to neutralize laboratory strains, but not primary isolates. The results of the screening were confirmed by further titrating some of the sera that showed significant neutralization, *i.e.*, > 75% neutralization of the virus. Table 6 summarizes representative data from serum titration experiments. In addition to non-neutralizing sera, sera were found that were narrowly neutralizing--*e.g.*, VRDB008 and VRDB018--whereas other sera were broadly neutralizing--*e.g.*, VRDB117.

The sera of the second category were recieved directly from MMCARR collaborators of Dr. Mascola--International Isolates--or from the World Health Organization--WHO. Figure 8 summarizes representative data from the International Isolate panels. In these, as previously reported by Dr. Mascola, sera seem to neutralize better within their genotypic clade.

The experiments using sera recieved from the WHO are part of another effort to serotype HIV. The data of Table 7 represent this laboratory's part in comparing two different typing methods: Serum Titration and Infectivity Reduction Assays--IRA. The data of Table 7 were compared with other data obtained in a collaborating lab (Dr. Peter Nara) and although there is good agreement between the results of the two methods, the IRA results are more ameable to statistical analysis and requires less work; representitive data from IRA titrations are presented in Table 8.

Virus Expansions and Evaluation of Cytopathic Effects

A substantial part of this contract's resources, particularly in the first two years, were alloted to the expansion and titration of viral stocks. In addition, investigations of the biological characteristics of viral isolates represents an important part of all clinical protocols supported by this contract. In addition to the existing procedures for virus expansion and titration, SRA established the ACTG's procedure for SI/NSI analysis in house in support WRAIR studies. These protocols can be found in the ACTG Virology Manual and, for that reason, will not be repeated. The numbers of expansions and evaluations of virus cytopathicity performed by SRA during the three base years of this contract are documented in the appendix.

Neutralization Assay Research and Development

A significant portion of this laboratories support of the 490 contract has been Vaccine Development. In collaboration with Dr. J. Mascola, a series of experiments have been conducted in the FY/94 contract year to better understand the results of neutralization assays; we have examined the kinetics of replication of several viruses, the effect and kinetics of neutralization prior to infection, the effect of neutralization concomitant with infection and neutralization after virus binding to cells was thought to occur.

It is well known that different viruses replicate at different rates. We confirmed the many previous observations of others and characterized 4 specific viruses. Characterization of different replication rates is directly relevant to the interpretation of subsequent neutralization experiments; *e.g.*, we need to know if the apparent decrease in replication of the virus in the presence of "neutralizing" antisera is merely an artifact of replication kinetics or true neutralization. The four viruses--two laboratory-adapted strains and two primary isolates--show wide differences in infection rates.

We have also found that laboratory viruses are neutralized more easily (or more quickly) than primary isolates prior to infection. In two separate experiments, virus and antisera were incubated for up to 16 hours prior to the addition of cells. After a standard infection period, the virus and antisera were removed and then viral replication allowed to proceed. The results of each experiment were the same: No statistically significant neutralization of primary isolates by the antisera used was measured in contrast with marked and time dependant neutralization of laboratory-adapted viruses.

In contrast to these results, both laboratory-adapted and primary isolates appear to be neutralized in a time-dependant manner. In several experiments, virus and cells were incubated for up to 16 hours prior to the addition of virus. After a standard "neutralization" period (1-2 h), the virus and antisera were removed and then viral replication allowed to proceed. The results of each experiment were the same: Statistically significant, time dependant neutralization of both primary and laboratory-adapted isolates by the antisera used was measured. The rates of neutralization differed for each virus and did not group according to source; *i.e.*, laboratory isolates are not neutralized faster or slower than primary isolates.

The results described suggest that laboratory-adapted viruses may be neutralized by an additional mechanism to that of primary isolates. The experiments performed to date are preliminary and have certain inherent limitations: Small sample sizes of both virus and antisera, suboptimal experimental control, and--in the earlier experiments--lack of the ability to statistical analyze the data because of experimental design. Using the virus infectivity reduction assay (described above) and newly available statistical evaluation software, we will be able to overcome these limitations.

Evaluation of Antiviral Gene Constructs

Gene therapy for immunological disorders, cancers and a variety of infectious diseases is quickly becoming a reality. This approach has been expanded from "simple" gene replacement or augmentation therapy to correct a genetic defect (as in the case of adenosine deaminase, ADA, deficiency [36,37] to new genetic treatments for

cancers[38] and infectious diseases such as AIDS.[39,40] There have been numerous proposals for the treatment of HIV infections using antisense genes [41-45] and genes containing catalytic RNAs (ribozymes).[46,47] *In vitro* interference with viral replication has been accomplished by targeting gene constructs to viral structural proteins[48-52], components of HIV's regulatory circuits[53-55] and the virus receptor, CD4.[56] The number of antiviral gene constructs available for testing appears to be multiplying exponentially.

Preliminary *in vitro* evaluation of these therapies has been accomplished, for the most part, in artificial systems sometimes employing biochemical endpoints or in well established cell lines using laboratory strains of HIV. Little is known about the efficacy of such treatments for primary isolates of HIV in normal human peripheral blood mononuclear cells (PBMC) and there are no published reports of quantitative determinations of putative antiviral gene effects on primary isolate-induced cytopathogenesis. Moreover, the impact of these constructs on the differentiation and ultimate immune function of human bone marrow derived hematopoietic stem cells, the apparent conveyance of choice for some gene constructs, is little understood. Finally, there still is no *in vitro* testing system available to bridge the gap between preclinical *in vitro* analyses and animal model systems such as the SIV model in macques.

In support of a WRAIR's gene therapy research and development, SRA was requested, in the first contract year (FY93) to develop *in vitro* assay systems to assess the efficacy of antiviral gene constructs against low passage, clinical isolates of HIV. Initial studies were to involve the use of syncytial-inducing isolates of HIV in established cell lines previously transfected with antiviral genes. This was to be followed by similar studies in PBMCs that would permit evaluation of a broader range of clinical isolates or, eventually, a prospective patient's own cells. The cell line chosen for the preliminary studies was MT-2, a line that is productively infected with HTLV-1, but is sensitive to infection by ≈35% of patient isolates. The initial studies conducted with these cells failed in the last fiscal year suggesting that production of HTLV-1 may have blocked the action(s) of the antiviral genes understudy. This virus could concievably interfer with expression, regulation or activities of the antivirals. New studies were conducted in FY94 using the A3.01 and SupT-1 cell lines, both of which are free of HTLV-1. Tables 9, 10, 11 and 12 illustrate the antiviral effects of a number of antiviral gene constructs using both reverse transcriptase and p24 endpoints in SupT-1 or A3.01 cell lines. Spurred by these sucesses we began to evaluate the possible use of purified CD4+ PBMCs in gene therapy by studying the susceptibility of PBMCs, stimulated with anti-CD28 and anti-CD3 antibodies, to virus infection. Our intention was to use cells, stimulated with these agents, and grown for extended periods *ex vivo*, as vehicles for the transduction of antiviral gene constructs. Our initial evaluation of this approach suggested that cells stimulated in this manner are not susceptible to infection with laboratory or clinical isolates of HIV. The section that follows details our investigations of this phenomenon and

Table 9 Effect of Antiviral Gene Constructs on HIV-1 Reverse Transcriptase Production											
Cell Line (SRA #)	pJM #	Description	CD4	Total Cell Count	Cells/Well	% of control growth	RT/Sample	Sample Vol.	RT/Well	RT/10e5 Cells	% Control
Virus = 8119											
CL094	Supt1	Parental line	+	7.85E+05	2.62E+05	100	2366667	100.00	473333	180892	100.00
CL097	GINA		+	7.45E+05	2.48E+05	95	647667	100.00	129533	52161	100.00
CL098	LRSN3		+	5.73E+05	1.91E+05	73	154618	23.87	30924	16205	31.07
CL099	LRSNDLTA78f		+	8.75E+05	2.92E+05	111	56249	8.68	11250	3857	7.39
Virus = RF											
CL094	Supt1	Parental line	+	7.85E+05	2.62E+05	100	3475000	100.00	695000	265605	100.00
CL097	GINA		+	7.45E+05	2.48E+05	95	536500	100.00	107300	43208	100.00
CL098	LRSN3		+	5.73E+05	1.91E+05	73	664833	123.92	132967	69677	161.26
CL099	LRSNDLTA78f		+	8.75E+05	2.92E+05	111	308667	57.53	61733	21166	48.99
Virus = IIIb											
CL094	Supt1	Parental line	+	7.85E+05	2.62E+05	100	2793333	100.00	558667	213503	100.00
CL097	GINA		+	7.45E+05	2.48E+05	95	493333	100.00	98667	39732	100.00
CL098	LRSN3		+	5.73E+05	1.91E+05	73	150840	30.58	30168	15809	39.79
CL099	LRSNDLTA78f		+	8.75E+05	2.92E+05	111	31518	6.39	6304	2161	5.44
CL097 is control for CL098 and 99.											

Table 10 Summary of Antiviral Effects of Gene Constructs in A3.01 Cells

Cell Line (SRA #)	pJM #	CD4	Total Cell Count	Cells/Well	% of control growth	p24/ml	% Control	p24/Well	p24 /10e5 Cells	% Control
490.GT0042	BK89	+	1.24E+06	4.12E+05	94	10	0.00	2	0	0.00
490.GT0043	321neo	-	9.23E+05	3.08E+05	70	1028	0.12	206	67	0.17
490.GT0044	321neo	+	1.52E+06	5.08E+05	116	547167	64.80	109433	21542	55.83
490.GT0045	357B	+	1.16E+06	3.86E+05	88	40909	4.85	8182	2121	5.50
490.GT0047	324	+	8.97E+05	2.99E+05	68	547072	64.79	109414	36593	94.84
490.GT0048	322neo	+	8.28E+05	2.76E+05	63	711000	84.21	142200	51522	133.53
490.GT0049	320neoA	+	1.02E+06	3.40E+05	78	1150148	136.22	230030	67656	175.35
490.GT0050	311	+	1.42E+06	4.72E+05	108	791833	93.78	158367	33529	86.90
490.GT0051	292neoA	+	1.21E+06	4.03E+05	92	497333	58.90	99467	24682	63.97
490.GT0052	160-2	+	1.06E+06	3.53E+05	81	2050000	242.80	410000	116038	300.75
490.GT0053	136	+	1.18E+06	3.94E+05	90	913333	108.17	182667	46323	120.06
490.GT0054	290	+	1.26E+06	4.20E+05	96	646448	76.56	129290	30759	79.72
490.GT0056	188	+	1.11E+06	3.68E+05	84	1171500	138.75	234300	63611	164.87
490.GT0057	253	?	1.21E+06	4.03E+05	92	1111	0.13	222	55	0.14
CL085	322	-	9.49E+05	3.16E+05	72	522	0.06	104	33	0.09
CL086	tat	+	1.05E+06	3.51E+05	80	71	0.01	14	4	0.01
CL087	362A	+	7.67E+05	2.56E+05	58	24118	2.86	4824	1887	4.89
CL088	362B	+	6.24E+05	2.08E+05	48	422	0.05	84	41	0.11
CL089	362A	+	5.72E+05	1.91E+05	44	3188	0.38	638	334	0.87
CL090	354	+	1.04E+06	3.47E+05	79	2426	0.29	485	140	0.36
CL091	tat	+	9.10E+05	3.03E+05	69	384500	45.54	76900	25352	65.71
CL093	A3.01	+	1.31E+06	4.38E+05	100	844333	100.00	168867	38583	100.00

Table 11 Evaluation of Antiviral Gene Constructs

Tech = Louis Davis
Virus = HIV-RF

Cell Lines =	**GT77**	**GT78**	**GT79**	**GT80**	**GT81**	**GT82**	**GT83**	**GT84**	**GT85**	**GT86**
			TCID50/TREATMENT							
REPLICATES	100	100	100	100	100	100	100	100	100	100
1	71486	952000	1240000	37717	48099	313000	312000	12	670000	294000
2	60436	821000	187000	78042	29438	1310000	52397	14	855000	122000
3	72825	1460000	711000	54706	23278	91182	45591	13	955000	486000
4	62932	888000	149000	50377	138000	152000	34528	13	749000	195000
5	21542	1370000	544000	27061	116000	209000	88667	13	876000	119000
No cell control	3015	32005	37819	1227	2374	22536	12472	9	119000	21734
MEAN	57844	1098200	566200	49581	70963	415036	106637	13	821000	243200
+/-SD	20981.99	294606.86	445341.11	19266.76	52543.41	506925.07	116581.71	0.71	111939.72	153273.29
CV	0.36	0.27	0.79	0.39	0.74	1.22	1.09	0.05	0.14	0.63
% CONTROL*	117	2215	1142	100	9	51	13	0	100	30

Virus = HIV - 8119

Cell Lines =	**GT77**	**GT78**	**GT79**	**GT80**	**GT81**	**GT82**	**GT83**	**GT84**	**GT85**	**GT86**
			TCID50/TREATMENT							
REPLICATES	100	100	100	100	100	100	100	100	100	100
1	129000	587000	1190000	35618	8552	100000	59421	24	55545	310000
2	41688	546000	402000	20254	14598	59654	>>>>>>>	22	64976	158000
3	20726	761000	315000	45082	22161	276000	172000	20	71446	311000
4	203000	579000	475000	40372	32288	331000	>>>>>>>	24	78011	650000
5	83574	715000	225000	70514	18334	60486	20169	29	67253	544000
No cell control	11468	17327	41062	1573	411	9264	2294	21	1919	9188
MEAN	95598	637600	521400	42368	19187	165428	83863	24	67446	394600
+/-SD	72985.55	94344.05	385321.03	18290.29	8879.34	128571.03	78811.38	3.35	8297.46	198541.68
CV	0.76	0.15	0.74	0.43	0.46	0.78	0.94	0.14	0.12	0.50
% CONTROL*	226	1505	1231	100	28	245	124	0	100	585

Table 12 Evaluation of Antiviral Gene Constructs

Tech = L. Davis
Virus = HIV-1RF

Cell Lines =	GT0096	GT0095	GT0094	GT0093	GT0092	GT0091	GT0090	GT0089	GT0088	GT0087
	TCID50/TREATMENT									
REPLICATES	100	100	100	100	100	100	100	100	100	100
1	7951	202000	93966	392000	133000	465000	794000	185000	1168	331
2	8332	195000	74147	568000	217000	295000	306000	>>>>>>>	>>>>>>>>	732
3	>>>>>>>	270000	19434	53310	138000	99319	190000	257000	871	>>>>>>>
4	>>>>>>>	216000	64277	>>>>>>>	>>>>>>>	432000	531000	318000	1231	427
5	>>>>>>>	404000	101000	584000	214000	165000	1160000	81600	827	387
No cell control										
MEAN	8142	257400	70565	399328	175500	291264	596200	210400	1024	469
+/-SD	269.41	87073.53	32173.92	246533.40	46249.32	160304.60	390659.95	101626.64	204.78	179.54
CV	0.03	0.34	0.46	0.62	0.26	0.55	0.66	0.48	0.20	0.38
% CONTROL*										

Virus = 8119

Cell Lines =	GT0096	GT0095	GT0094	GT0093	GT0092	GT0091	GT0090	GT0089	GT0088	GT0087
	TCID50/TREATMENT									
REPLICATES	100	100	100	100	100	100	100	100	100	100
1	222000	62025	565000	169000	474000	734000	878000	1160000	91	991
2	57187	156000	201000	224000	501000	477000	839000	651000	538	1972
3	15103	103000	11392	>>>>>>>	907000	261000	725000	441000	781	>>>>>>>
4	50129	19831	207000	>>>>>>>	708000	577000	1000000	587000	380	>>>>>>>
5	186000	113000	40278	239000	646000	1050000	764000	837000	486	>>>>>>>
No cell control										
MEAN	106084	90771	204934	210667	647200	619800	841200	735200	455	1482
+/-SD	91682.21	51858.74	220361.85	36855.57	175047.71	295412.76	107283.27	276778.25	251.12	693.67
CV	0.86	0.57	1.08	0.17	0.27	0.48	0.13	0.38	0.55	0.47
% CONTROL*										

describes studies that have led to the discovery of soluble inhibitory factors in the supernatants of CD3/CD28 antibody-stimulated CD4+ T-cells.

Identification of Putative Anti-HIV factors in Long Term Cultures of CD4+ T-Cells Intended as Targets for Anti-Viral Gentic Therapy

A process for the *in vitro* expansion of CD4+ T-lymphocytes obtained by apheresis of normal donors or HIV-infected patients has been developed in Dr. Carl June's laboratory that utilizes partially purified PBMCs or purified CD4 cells co-stimulated with antibodies to CD3 (T3) and CD28 (9.3) covalently linked to paramagnetic microbeads. The cell population resulting from this stimulation is CD4+ with no apparent contaminating monocytes, macrophages or CD8 cells. Peripheral mononuclear cells from nine HIV-infected patients have been cultured to date and maintained thru multiple generations, some in excess of 100 days. Cell numbers have grown from 10^5 at the start of culture to, in some cases, well over 10^{12} at culture termination.

These cells are intended as targets for ex-vivo modification by antiviral therapeutic genes or drugs followed by re-infusion into their autologous hosts. An important component of these investigations is the pre-infusion testing of antivial gene efficacy *in vitro*. Most efficacy analyses of antiviral gene constructs are conducted in established cell lines with laboratory adapted strains of HIV. Evaluation of antiviral therapies with clinical isolates in established cell lines is limited to but 35% of isolates (syncytial inducing), usually obtained from individuals late in their course of disease. Effective determination of efficacy utilizing primary isolates of HIV in normal donor PBMC has been difficult because of the short term nature of PBMC cultures and problems arising from the efficiency of transduction and selection of target cells. The effective longterm culture of PBMCs from normal donor and HIV-infected patients by combined a-CD3/a-CD28 (T3/9.3) stimulation obviates the first of these problems, but treatment efficacy analysis requires these cells (when employed as no treatment controls) to be sensitive to virus infection. Previous studies indicated that stimulation of PBMCs with soluble T3/9.3 antibodies results in the activation of latent HIV infection (3) in patient cells and predicts that normal donor T-cell sensitivity to HIV infection should result from such stimulation. The present investigation was initiated, in part, to develop a system for the efficacy analysis of antiviral gene constructs produced by WRAIR scientists and, in part, to confirm T3/9.3-induced, CD4+ T-cell, sensitivity to HIV infection. We report here the rather surprising result that T3/9.3 solid phase (in cis) stimulation yields a cell population that is reversibly resistant to HIV infection or transmission and actively transmits this suppression, by way of a soluble factor(s), to cells normally susceptible to infection.

To evaluate the susceptibility of long term PBMC cultures to HIV we attempted to

infect normal donor cells, stimulated with T3/9.3 (cis) microbeads, with a US (clade B) clinical isolate of HIV-1 (9881) isolated, expanded and titrated in PBMCs. Normal donor PBMCs were stimulated for three days with either PHA or T3/9.3 covalently linked to paramagnetic microbeads and compared for sensitivity to HIV infection. The results of this study (Table 13) suggested that T3/9.3 stimulation prevented or depressed HIV infection while cells from the same donor, stimulated with PHA, were fully susceptible to infection. No virus p24 production was detected in culture supernatants derived from T3/9.3 stimulated cells through day 21 in the presence of the T3/9.3 microbeads, while PHA-stimulated cells produced 18,820 pg of p24 by day 7 and more than 65,000 pg by day 14. Microbeads, lacking T3/9.3 antibodies were not inhibitory in this study (data not shown). Subsequent investigations (data not shown) showed that the T3/9.3 stimulated cells were not devoid of the CD4 receptor for HIV and HIV infection of these cells did not depend on an altered requirement for polybrene pretreatment.

Several questions are raised regarding this inhibition including: 1) whether the microbeads in some way interfer directly with infection, 2) whether inhibition is unique to the leukopack or virus in question or 3) to the method of stimulation, 4) to the mechanism of inhibition, 5) to the cell populations involved and finally, 6) whether inhibition of HIV infection is reversible. To address these issues we performed a series of studies with additional leukopack-derived cells from different normal donors. Ficoll purified PBMCs were stimulated with T3/9.3 on microbeads. After three days the microbeads were removed from half the cells by centrifugation on ficoll gradients, portions of these bead-free cells were either reincubated or stimulated with PHA. Portions of the remaining, bead-free "resting" cells were similarly stimulated with PHA on days 6 and 10. All PHA stimulated cells along with their unstimulated and T3/9.3 antibody bead containing controls and were infected with 100 $TCID_{50}$s of HIV-19881 and evaluated seven days later for the presence of culture supernatant p24. These "rested" cells were then restimulated, but with PHA alone, and three days later compared with T3/9.3 stimulated cells (with beads present throughout) for sensitivity to 100 $TCID_{50}$s of HIV-1_{9881}. T3/9.3 stimulated cells, with beads present or from which antibody containing beads had been removed produced no detectable or barely detectable supernatant p24 throughout the 10 day experimental period (Table 14). PHA stimulation of T3/9.3 stimulated, bead-free cells produced no detectable supernatant p24 until they rested for at least 6 days. These results suggest that suppression of HIV infection by stimulation with antibodies to T3/9.3 anchored on paramagnetic microbeads beads is reversible and offers the possibility of establishing long-term cultures and large repositories of CD4+ cells from individual normal donors. This would reduce assay-to-assay variability and enhance reproducibility for many HIV test systems. In similar studies, infection with HIV-1_{BAL}, a monocytropic variant, was also inhibited in PBMC or CD4+ T-cell cultures reinforced with 10% monocyte/macrophages and stimulated with T3/9.3 microbeads (Table 15). Studies with other clinical isolates of HIV-1 are underway to

Table 13
HIV-1 Virus Titrations in CD3/CD2 and PHA Stimulated PBMCs

	TCID50		
Cell Group	*Day 7*	*Day 14*	*Day 21*
CD3/CD28 Sti	<64*	<64	<64
PHA Stim.	18820	>65000	>65000

Cells infected with US 1 Isolate
Culture supernatants evaluated for p24 on the indicated days
*TCID50 Determined by the method of Spearman-Karber

Table 14
Effect of CD3/CD28 Mitogen Removal on Sensitivity of Patient Cells to HIV Infection

Cell Group	Description	Day of Mitogen Removal		
		3	6	10
1	734714 , CD3/	135	64	55
2	737414, Beads	90	39	64
3	734714, PHA	<1000	>50000	>250000

Table 15
Virus Titrations in PHA and CD3/CD28 Stimulated PBMCs With or Without Monocyte/Macrophages

	US1 TCID50		*BAL TCID50*
Cell Group	*Day 7*	*Cell Group*	*Day 7*
PBMC+PHA	332555	PBMC+PHA	241029
OKT3/9.3 Bea	279	OKT3/9.3 Bea	77
CD4,M/M+PHA	>390625	CD4,M/M+PHA	>390625
CD4,M/M+Bea	386	CD4,M/M+Bea	279
CD4 + Beads	1012	CD4 + Beads	386
SRA PBMC Cor	241029	SRA PBMC Cor	174693

Culture supernatants evaluated for p24 on the indicated days
TCID50 Determined by the method of Spearman-Karber

Table 16

Effect of T3/9.3 Stimulated Cells on Virus Infection in PHA Stimulated Cells

	Number of Cells		p24 with PHA PBMC:	p24 with PHA PBMC:	
Cell Group	CD3/CD28	PHA PBMC	no added CD3/CD28 Cells	+ added CD3/CD28 Cells	% of PBMC Control p24
1	0	200000	68063	-	100
2	20000	160000	93076	2193	2
3	40000	120000	96131	1617	2
4	80000	80000	77666	521	1
5	120000	40000	48404	234	0
6	160000	20000	25564	75	0
7	200000	0	-	194	-

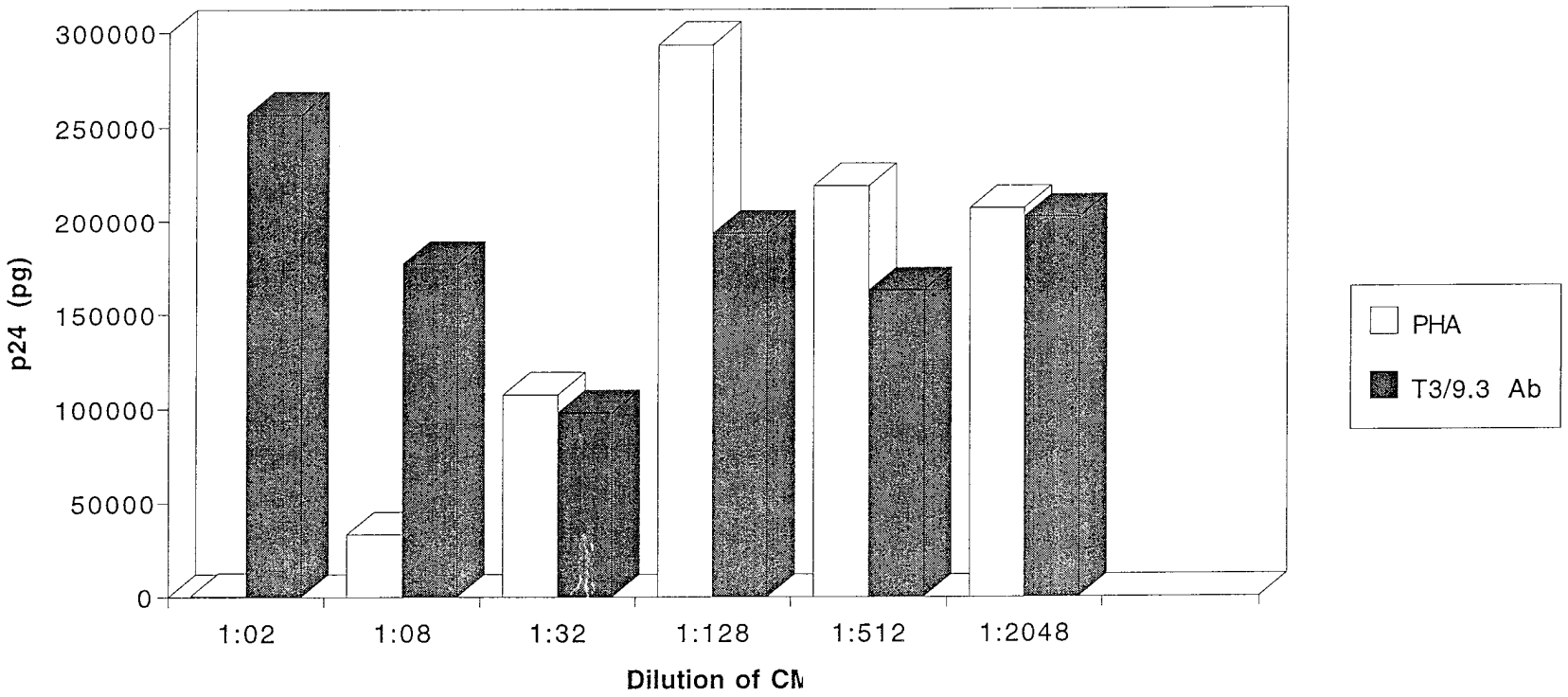
Table 17
EFFECT OF CONDITIONED MEDIA FROM T3/9.3-STIMULATED CD4 CELLS ON HIV-1 INFECTION IN PHA-STIMULATED PBMCs

CELL GROUP	Supernatant p24*	% Inhibition	=/-SD	CV
PBMCs/PHA+IL2	16373		14996	0.916
PBMCs/PHA+IL2+50%CM(PHA Stim)	41080	0	35731	0.870
PBMCs/PHA+IL2+50%CM(T3/9.3 Stim)	1285	92	716	0.557

Table 18
Effect of Conditioned Media From CD3/CD28 Stimulated Cells on Sensitivity to HIV Infection

Cell Group	TCID50	Percent Reduction
PBMCs+PHA (Autologous Donor)	91767	--
PBMCs+PHA (Heterologous donor)	66511	--
PBMCs+Fresh Cond. Autologous Sup.	3671	96
PBMCs+Frozen Cond. Autologous Sup.	18353	80
PBMCs+Fresh Cond. Heterologous Sup.	18353	72

Figure 9 Conditioned Media Titrati



confirm the general applicability of this phenomenon.

A series of experiments were directed toward determining whether inhibition of infectivity by T3/9.3 results from active suppression by stimulated cells or their lymphokines. Purified CD4+ T-cells were washed free of T3/9.3 linked microbeads after 3-4 days of stimulation. Dilutions of these cells were then mixed with PHA-stimulated PBMCs and the cocultures infected with HIV-1_{9881} and evaluated seven days later for supernatant p24 as described in the methods. PHA-stimulated PBMCs alone produced 68,000 pg of p24/ml of supernatant while T3/9.3-stimulated cells alone produced only 194 pg/ml in seven days. HIV p24 synthesis was inhibited 98% in cocultures containing as little as 10% T3/9.3 - stimulated CD4+ cells (Table 16).

Subsequent experiments have suggested that this inhibition is the result of a soluble factor(s) present in supernatants derived from the T3/9.3 - stimulated CD4+ cell cultures. Conditioned media, derived from either T3/9.3 - stimulate, but not PHA-stimulated PBMCs or CD4+ cells 3-4 days following stimulation supressed production of supernatant p24 in PHA-stimulated PBMCs or CD4+ target cells (Table 17). Additional studies, employing 50% conditioned media suggested a marked inhibition of HIV infection in both autologous and heterologous cells (Table 18). Frozen supernatants were capable of inducing inhibition in PHA stimulated cells from both autologous and heterologous cells, but to a somewhat lessor extent when compared with fresh autologous supernatants. Figure 9 illustrates the results of a titration study comparing conditioned media from PHA- and T3/9.3 stimulated cultures. The data suggest that a factor or factors, present in conditioned media obtained from T3/9.3-, but not PHA-stimulated cells inhibits the infection, transmission or expression of HIV in a dose dependent fashion. It is hoped that this work will continue under a CRADA developed in collaboration with WRAIR and SRA. This effort will be directed toward the isolation, identification and characterization of any HIV inhibitory factors which might be present.

3. Antiviral Drug Testing Group

The principal focus of the antiviral drug testing group is the utilization of existing *in vitro,* peripheral blood mononuclear assays to determine drug susceptibility of patient isolates in support of WRAIR clinical protocols. In addition, this unit performs phenotypic analyses for drug resistance and has developed, in collaboration with Dr. Mayers a rapid screening assay for identification of drug resistance at the time of virus isolation. The protocol for this is described below. Finally, the drug testing group has the responsibility to evaluate putative antiviral agents developed by WRAIR scientists. During the period 10/1/92-9/30/95 the drug testing group supported the following studies:

1. RV43 - to determine the incidence and clinical significance of AZT resistance

in patients with HIV disease being treated with AZT.

2. RV65 - to determine the time course of development of resistance to an experimental compound (here called compound A) in patients with HIV isolates demonstrating *in vitro* resistance to AZT.

3. RV79 - An ACTG/NIAID sponsored clinical evaluation of the codon 215 genotypic assay. To date more than a hundred specimens have been evaluated for the presence or absence of this phenotype.

4. CPCRA (007/014) - prospective evaluation of the development of *in vitro* anti-retroviral resistance in HIV-1 isolates obtained from patients participating in the CPCRA Combination Nucleoside clinical trial.

5. The Johns Hopkins University/MACS studies.

6. The Johns Hopkins University seroconverter study.

7. The *in vitro* testing of experimental anti-retroviral compounds using HIV-1 isolates.

The drug sensitivity assays performed on RV43 and RV65 isolates resulted in the determination of the *in vitro* drug inhibitory concentration of four anti-viral agents for each virus isolate tested. An example of a final report for an RV43 patient is presented in Table 19. During this period 268 drug sensitivity assays were performed and reported for RV43 patient isolates. Assays performed on the six patients enrolled in the RV65 study examined the *in vitro* resistance to AZT, ddC, ddI, and compound A. Thirteen assays were performed before this study was terminated.

For the additional studies, 187 CPCRA specimens were received and processed for virus isolation. It is anticipated that during the next fiscal year virus titration and drug sensitivity assays will be performed on these isolates. For the Johns Hopkins University MACS study the drug testing group received 52 vials of frozen cells from individual patients for virus isolation. Virus was isolated from 30 of these specimens and we were requested to determine the virus titration and *in vitro* resistance to AZT for 16 of these isolates.

For the Johns Hopkins University seroconverter study, we received 16 isolates for

Table-16: VIRUS ISOLATE NUMBER 102743

Date Received: 3/4/94

Date Virus Titration Set-up: 5/8/94

Titration Data

Assay	O.D.	Number of + wells per Virus Stock								4-Drug Plate Virus Dilution
Date	Cutoff	16	64	256	1024	4096	16384	65536	$TCID_{50}$	Required
5/19/94	0.504	6	6	6	6	2	2	1	6472	0.530

Date Drug Sensitivity Set-up: 6/1/94

Date Drug Sensitivity Assayed: 6/8/94

Drug Sensitivity Data

AZT (uM)	$p24x10^5$	Fraction Affected	ddC (uM)	$p24x10^5$	Fraction Affected
0	3.69		0	3.69	
0.001	3.35		0.01	2.83	0.23
0.01	3.44		0.1	0.48	0.87
0.1	3.52	0.05	1.0	0.04	0.99
1.0	2.01	0.46			
5.0	0.25	0.93			

IC_{50} = 0.8979

IC_{50} = 0.0247

ddI (uM)	$p24x10^5$	Fraction Affected	Compound A	$p24x10^5$	Fraction Affected
0	3.69		0	3.69	
0.1	3.33	0.10	0.03	3.63	0.02
1.0	2.86	0.22	0.3	0.09	0.98
5.0	2.46	0.33	1.0	0	
10.0	1.40	0.62	3.0	0	
25.0	0.07	0.98			

IC_{50} = 2.5091

IC_{50} = 0.0949

esting *in vitro* AZT resistance. Because of low virus titration results obtained for two of these isolates, assays could only be performed on 14 of these specimens.

Several experimental compounds were tested to determine their ability to inhibit replication of HIV isolates. These drugs were obtained from the laboratories of the Department of Applied Biochemistry of the Walter Reed Army Institute of Research, the Laboratory of Medicinal Chemistry at the National Cancer Institute, and private pharmaceutical companies. These compounds were tested by using isolates from RV43 patients and AZT-resistant and sensitive control virus isolates

Drug Testing (10/1/92 - 9/30/95)

During this period, specimens were received and processed for RV43, CPCRA and RV79 as described above. The processing of these samples represented the establishment of more than 800 tube cultures for isolation and ultimately expansion. A summary of samples processed is presented in the Appendix.

Development of a rapid drug screening assay

The current assays used to identify drug resistant HIV isolates require virus isolation, expansion and titration of the isolate followed by phenotyping. In order to reduce the time and cost, a rapid drug phenotype screening assays was developed. After a positive virus culture is confirmed, the procedure is as follows:

1. Resuspend the cells in the p24 positive culture (A1 Tube) and divide into three tube cultures each containing 0.800 ml of the resuspended cells. Label the tubes with SRA number and as AZT 0, AZT 0.2, or AZT 2.0

2. Into each of the three tubes, place 0.450 fresh co-cult media containing $2x10^6$ PHA-stimulated PBMCs.
3. Into the tube labelled AZT 0, place 1.25 ml fresh co-cult media. Into the tube labelled AZT 0.2, place 1.25 ml fresh co-cult media containing 0.4 uM AZT. Into the tube labelled AZT 2.0, place 1.25 ml fresh co-cult media containing 4.0 uM AZT. Final volume of all three tubes should be 2.5 ml.

4. Continue to maintain the cultures using standard procedures. On Day 4 replace the media in the culture tubes with either co-cult media, co-cult media with 0.2 uM AZT, or co-cult media with 2.0 uM AZT. On Day 7, refeed the cultures with $2x10^6$ PHA-stimulated PBMCs in the appropriate media, i.e. no AZT, 0.2 uM AZT, or 2.0 uM AZT. Save an aliquot of media from each tube on Day 4 and Day 7 refeeds for p24 assay.

   The p24 (pg/ml) results of this assay using 22 isolates and comparison values to the conventional assay is shown in Table 23.

conventional assay is shown in Table 23.

Table 23

Isolate	AZT Concentration (uM) 0	0.2	2.0	IC50 (uM) by ACTG/DoD
1	1024	0	0	0.1010
2	1136	0	0	0.0920
3	867	321	0	1.3947
4	1467	416	0	4.4568
5	1102	0	0	0.0206
6	1445	56	0	0.6692
7	1876	827	47	1.4245
8	1203	528	0	1.5471
9	2046	1002	116	2.3359
10	1422	0	0	0.1371
11	1127	0	0	0.0379
12	1876	18	0	0.1767
13	1443	0	0	0.0603
14	1154	139	0	0.5008
15	1889	477	0	0.2527
16	1322	264	18	0.5843
17	964	567	43	0.3595
18	1221	1316	867	>5.0000
19	1802	1765	675	4.4568
20	1556	556	110	1.6692
21	1677	627	97	2.6602
22	1765	760	119	1.3726

4. Data Group Section

The computer capabilities of contract DAMD17-92-C-2504 took a major leap forward during the three base years of this contract with the installation of a wide-area network encompassing, first, the Taft Court, Shady Grove, and Key West facilities of SRA and finally, subsequent to consolidation, a fully networked facility at 4 Research Ct. This process involved several steps, including upgrading of the RLIMS database hardware, installation of linked local area networks (LANs), provision of 386-type or better computers for all personnel and provision of new software packages. In addition, Direct communications between the Henry Jackson foundation computers were established on an interim bases for the uploading of data from RLIMS and a ccMail connection was put in place to facilitate direct communications between WRAIR and SRA personnel.

Establishment of Information System Hardware

The RLIMS database upgrade step involved replacement of an aging 386-based 8-user SCO Unix-based host with a Sun SPARCserver 670, capable of supporting upwards of 100 users and accommodating 5 years of data growth. The response time of queries on the system improved 1000% in many instances. The data support group was consolidated at the Key West facility, improving internal efficiency. Programming productivity has increased enormously due to the new high-speed equipment.

The second step involved linking the three sites internally with LANs and connecting those LANs via T-1 digital phone lines and microwave radio. Standard Windows-based applications were installed for universal access, including WordPerfect, Excel, and cc:Mail. Now, with the exception of the Macintosh, a user can perform any task from any workstation at any site. New computers were acquired for key personnel to replace 286 PCs, and eight computers were acquired for pooled use by lab technicians.

Introduction of New Software

1. Oracle Database - The Oracle database software was upgraded from Oracle v6 to ORACLE7. This improved data integrity and security and improved programming by enabling much of the data validation and processing logic to be coded into the database rather than the applications. SRA's Relational Laboratory Information Management System (RLIMS) utilizes Oracle and now resides on a Sun SPARC server located at 4 Research CT. The RLIMS Database contains patient identifiers, specimen information, freezer inventory, and results associated with the contract. The benefit of the RLIMS database being on the network is the ability for end users to access information concerning specimens they are assigned via Forms or Reports that reside on the RLIMS system.Investigation began into the acquisition of new graphical Windows/Mac front-end software to replace the character-mode Unix interface, and graphical LAN Architecture-end-user query software to enable scientists and lab technicians to build their own reports from the database without programming.

2. Excel Spreadsheet - Excel is the standard spreadsheet software package used on SRA's Lab Network. This package permits Lab supervisors as well as the technicians to analyze raw data produced by experimentally and stored in RLIMS. Currently raw data is either typed in or downloaded directly into a spreadsheet template for calculation or statistical evaluation. Once the spreadsheet is complete report formats including graphical charts can be applied and distrubuted through out the group via file sharing or our E-mail package CCMail.

3. CC Mail - This Software package allows contract personnel to communicate within their group and with others users on the network. This e-mail

capability permits distribution of experimental protocols and data to all technical staff and interested parties without resorting to scheduled meetings. CCMail also supplies a central point for technicians to store messages and files associated with tasks assigned by the laboratory supervisors. The Data Group worked closely with various SRA contract personnel to create procedures and programs within RLIMS to handle experimental plate data for all experimental work. The Data Group wrote a number of reports, and formulated methods to e-mail RLIMS output to contract personnel in Excel format.

It should be noted that all software packages were upgraded in FY95 to include WordPerfect 6.0/6.1 for PC and 3.5 for Mac, Excel 5.0 for both platforms and the latest cc Mail which now accomodates internet communications. Full internet access was also established in FY95 with the introduction of Netscape software and the construction of SRA's own home page on the world wide web. The homepage is still in the process of revision and should be completely functional in the early part of FY96.

Finally, at the request of WRAIR personnel and the contract office's representative, a procedure was established to pass all contract generated data to WRAIR via the Henry M. Jackson Foundation's (HMJF) computer network. The first data transfer encompassed the period January 1988 through July 1993. HMJF loaded the data into their Informix database for further processing at the discretion of WRAIR investigators. The data was transferred on 1/4" tape. Meetings were held to formulate plans for direct linking of the new SRA network with the WRAIR/HMJF computer network, allowing HMJF to access approved data downloads directly. HMJF agreed to procure and install the necessary equipment. Since SRA's consolidation, some changes in transfer procedures were required and the T1 line was terminated. Finally, new programming capability has been added and staffing increased to accomodate the workload.

All told, during the period encompassed by this contract (10/1/92-9/30/95) SRA has spent close to one million dollars on an upgraded computer facility which has significantly improved communications and data flow between WRAIR and SRA principal investigators.

SUMMARY

A number of significant contributions to WRAIR's mission were made by this contract from Oct. 1992 thru the end of Sept. 1995. Included is the optimization, validation and extension of the 215 ARMS assay for genotypic resistance patients in a large-scale, nationwide clinical trial to assess the significance of this mutation. Comparison of the genotypic approach with the classical phenotypic assays of drug resistance described in the previous sections will undoubtedly demonstrate the usefulness of the genotypic assay for clinical management of patient therapy. During the contract period SRA also developed an ARMS assay for codon 74 mutations associated with DDI resistance. In addition to the qualitative 215 assay currently available, SRA is working to establish a procedure for the quantitative determination of RT mutations so as to

discriminate mixtures and thus enhance the physcians ability to identify patient requirements for treatment modifications. It is hoped that such an assay will be available sometime in FY96.

In addition to the mutational assay, SRA put in place manual sequencing procedures for HIV reverse transcriptase, developed primers and procedures for env sequencing and, thru WRAIR's collaboration with the AIDS Clinical Trials Groups, began efforts to bring protease sequencing online. During the last fiscal year, a time when funding was sharply reduced and the program was itself in jeopardy, SRA established an automated sequencing facility to enhance the capabilities already available to the government. These capabilities will permit WRAIR to evaluate resistance mutations and determine their significance on a patient-by-patient basis regardless of the test drugs employed.

Finally, in the molecular area, the analysis of viral burden has become the principle surrogate marker of drug efficacy. A number of assays, commercial and in-house have been offered for quantitation of this marker including Roche's Amplicor, NASBA, bDNA, etc. At the request of the COR, SRA became certified to run the Roche assay and has trained two staff members who have been validated by Roche and the ACTG to provide this service. SRA now provides RT-PCR quantitation for diagnostic work performed by WRAIR for its patients. In FY96 SRA will be training additional personnel to ensure continued support for this area of research.

SRA, thru the cell phenotype working group provided services for the isolation, expansion and titration of isolates employed for serotyping and neutralization. Although demand for this service has declined, because of cost considerations, it remains available to all WRAIR investigators. As a result of the problems in funding the cellular phenotype working group has shifted emphasis to studies of gene therapy. We have developed an *in vitro* assay for the evaluation of antiviral gene constructs in established cell lines and is actively pursuing a system that will permit efficacy analysis in PBMCs with primary isolates. Such a system will prove invaluable for the longterm culture and ex-vivo treatment of patient cells with antiviral genes. Of particular note is the rather surprising discovery, made at SRA in collaboration with investigators at the Navy's Medical Research Institute in Bethesda, that T3/9.3 solid phase (in cis) stimulation yields a cell population reversibly resistant to HIV infection or transmission and and that this cell population actively transmits this suppression, by way of a soluble factor(s), to cells normally susceptible to infection. This work suggests the existence of a heretofore unknown mechanism of resistance to HIV infection and points toward new methodolgies for the treatment of infected individuals. This represents a major contribution by WRAIR and SRA to the immunobiology and therapy of HIV. For FY96 work is continuing on the supernatant factors, obtained from CD3/CD28 stimulated cell culures, responsible for inhibition of HIV infection. Studies planned and in progress include scaleup of supernatants, isolation, identification and characterization of these factors.

During the three base years of this contract the drug testing group supported a large number of clinical protocols including RV43, RV65, RV77 and RV79 in addition to the ACTG's CPCRA studies. Again because of funding reductions, the drug testing group sharply reduced efforts to test newly develped putative antiviral agents. However, support for a number of protocols,

requiring drug susceptibility phenotyping have continued and SRA participates with this contracts COR in the ACTG's drug resistance SWAT teams and have been instrumental in the development of an improved system for the rapid and cost effective phenotypic analysis of resistance to antiviral drugs as described earlier in this report.

Finally, SRA has made great strides in its continuing effort to provide computer support and data communications to WRAIR and its collaborators. Almost one million dollars has been spent to improve SRA's computing infrastructure and upgrade its programing staff.

The contractor is currently on a one year no cost extention. Assuming availability of funding, it is hoped that the contracted option years will be excercised by the government. A number of ongoing studies and protocols would be impacted otherwise. Plans for support of clinical trials for private companies and the NIH who will providing funding to the program have been put in place.

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APPENDIX

I Publications and Abstracts for FY95, FY94 and FY93

Papers

1. Anderson DW, DeNobile J, Zhao F, Vahey M, **Lane J**, Nau M, Brown AE, Malone JD, Wagner K, Ghosh B, Cotelingam JD, Mayers DL: Sampling lymph node content of human immunodeficiency virus-1 nucleic adicds and p24 antigen by fine needle aspiration in early stage patients. (Accepted, AAC), 1995.

2. Brown AE, Vahey MT, Zhou S, Chung R, Ruiz NM, Hofheinz D, **Lane JR**, Mayers DL and the RV-43 Study Group: Quantitative relationship of serum p24 antigen with plasma HIV RNA and p24 antibody in HIV-infected subjects on antiretroviral therapy. J Infect Dis, 172:1091-1095, 1995.

3. Buckheit RW, Fliakas-Boltz V, Yeagy-Bargo S, **Weislow O**, Mayers DL, Boyer P, Hughes S, Bader JP: Resistance to 1-[2-hydroxyethoxy)methyl]-6-(phenylthio)thymine is generated by mutations at multiple sites in the HIV-1 reverse transcriptase. Virology, 210(1):186-93, 1995.

4. Chiang P, McCann P, **Lane J**, Pankaskie M, Burke D, Mayers D: Anti-human immunodeficiency virus (HIV-1) activities of inhibitors of polyamine pathways. (Submitted), 1995.

5. Mayers DL, Mikovits JA, Joshi B, Kewlett IK, Estrada JS, Wofe AD, Garcia GE, Doctor BP, Burke DS, Gordon RK, **Lane JR**, Chiang PK: Anti-human immunodeficiency virus (HIV-1) activities of 3-deazaadenosine analogs: Increased potency against 3'-azido-3'-deoxythymidine-resistant HIV-1 strains. PNAS, 92:215-219, 1995.

6. Mascola JR, **Snyder SS, Weislow OS**, et al. Vaccination with Envelope Glycoprotein Products Induces Neutralizing Antibodies to Laboratory-Adapted but not Primary Isolates of HIV-1. J Infectious Dis (In Press)

7. Wegner SA, Anderson DW, DeNobile J, Cotelingam JD, **Lane J**, Nau M, Zhao F, Vahey MT, Mayers DL: Pilot study of rectal mucosal biopsy as a means of assessing lymphoid tissue in patients with early stage HIV-1 infection. (Accepted, JID), 1995.

8. Mayers DL, Mikovits JA, Joshi B, Hewlett IK, Estrada JS, Wolfe AD, Garcia GE, Doctor BP, Burke DS, Gordon RK, **Lane JR**, and Chiang PK. Novel anti-HIV-1 activities of 3-deaza-adenosine analogs: increased potency against azt-resistant HIV-1 strains. Proc. Nat. Acad. Sci.: Accepted for publication, 1994.

9. Mayers DL, Japour AJ, Arduino JM, Hammer SM, Reichman R, Wagner KF, Chung R, . **Lane J**, Crumpacker CS, McLeod GX, Beckett LA, Roberts CR, Winslow D, Burke D: Dideoxynucleoside resistance emerges with prolonged zidovudine montherapy, Antimicrobial Agents and Chemotherapy, 38(2):307-314, 1994.

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12. Japour, A.J., Mayers, D.L., Johnson, V.A., Kuritzkes, D.R., Beckett, L.A., Arduino, **J., Lane**, J., Black, R.J., Reichelderfer, P.S., D'Aquila, R.T., Crumpacker, C.S., The RV-43 Study Group and The AIDS Clinical Trials Group Virology Committee Resistance Working Group, Standardized Peripheral Blood Mononuclear Cell Cultrue Assay for Determination of Drug Susceptibilities of Clinical Human Immunodeficiency Virus Type 1 Isolates. Antimicrobial Agents and Chemotherapy, 37(5):1095-1101, 1993.

Abstracts

1. Anderson DA, Denobile J, Vahey M, Zhao F, Lane J, Nau M, Wegner SA, Wagner KF, Ghosh B, Cotelingam JD, Mayers DL. Potential for lymph node fine needle aspiration to sample virologic markers in early stage HIV Patients. 2nd National Conference on Human Retroviruses and Related Infections, Washington, DC, 1995.

2. Anderson DA, Nau M, Lane J, Brown A, Wegner SA, Wagner KF, Cotelingam JD, Mayers DL. Lymphoid tissue viral load and histologic pattern in early, mid and late stage HIV-1 patients. 2nd National Conference on Human Retroviruses and Related Infections, Washington, DC, 1995.

3. Bernstein, W.B., St. Louis, D.C., Perfetto, S., Weislow, O., Chung, R.C.Y., Wagner, K.F., D.L. Mayers. The timing and development of Codon 215 mutations in plasma, CD4 and CD14 cells from HIV infected patients on Zidovudine, 2nd National Conference, Human

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5. Mayers DL, Yerly S, Perrin L, Imrie A, Cooper DA, Karney WW, Brown AE, Rakik A, Harris R, Gambel J, Weislow OS, Lennox JL, Burke DS: Prevalence and clinical impact of seroconversion with AZT-resistant HIV-1 between 1988 and 1994. 2nd National Conference on Human Retroviruses and Related Infections, Washington, DC, 1995.

6. Mosca JD, Kaushal S, LaRussa V, Kessler S, Gartner S, Kim J, Perera P, Yu Z, Ritchey D, Xu J, Rosenberg Y, St. Louis D, Weislow O, Mayers D, Burke D: Human bone marrow-derived CD34+ cells as targets for gene therapy against HIV infection. 2nd National Conference on Human Retroviruses and Related Infections, Washington, DC, 1995.

7. Mayers DL, Lane J, and Weislow OS. Rapid screen of clinical specimens for drug resistant HIV phenotypes during virus isolation. Third International Workshop on HIV Drug Resistance, Kauai, Hawaii, August 1994.

8. Mayers DL, Yerly S, Perrin L, Imrie A, Cooper DA, Karney WW, Brown AE, Rakik A, Harris R, Gambel J, Weislow OS, Lennox JL, and Burke DS. Prevalence and clinical impact of seroconversion with AZT-resistant (AZTR) HIV-1 between 1988 and 1994. Second National Conference on Human Retroviruses and Related Infections, December 1994, Washington D.C.

9. Mayers DL, Wagner KF, Chung RCY, Vahey MT, Weislow OS, Zhou S, Burke DS, RV43 Study Group: Virologic Response to ddI following prolonged AZT Rx. Xth International Conference on AIDS, Japan, Abstract 359B, 1994.

10. Mayers D, Wagner KF, Chung RCY, Vahey MT, Weislow OS, Lennox JL, Zhou SYJ, Burke DS: Realtionship of SI/NSI status to development of ZDV resistance and clinical outcomes in patients on ZDV therapy. HIV Drug-Resistance Third International Workshop, Kauai, Hawaii, 1994.

11. Mascola JR, Weislow O, Snyder S, et al. Comparison of Genetic Subtype to Neutralization Serotype for Human Immunodeficiency Virus Type-1. (1994 Keystone Symposia: Prevention and Treatment of AIDS.) J Cell Biochemistry 1994;18B [Abstract J321].

12. Mascola JR, Weislow O, Snyder S, et al. Neutralizing Antibody Activity in Sera from Human Immunodeficiency Virus Type-1 Vaccine Recipients from the AIDS Vaccine Clinical Trials Network. VIth Annual Conference on Advances in AIDS Vaccine

Development (1993 NCVDG). AIDS Res Hum Retroviruses. 1994;10 (Supp 2):S55.

13. Robb ML, Frey S, Polonis V, Mayers DL, Ruderman J, White FA, Lane J, Michael NL, Turnicky R, Redfield RR: Comparison of HIV-1 env and pol evolution in lymph node, peripheral blood and plasma. Xth International Conference on AIDS, Japan, Abstract 524A, 1994.

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15. Chiang, P.T., Joshi, B., Hewlett, I.K., **Lane, J.R.**, Doctor, B.P., Burke, D.S. and Mayers, D.L., 3-Deaza Adensosine Analogs as Novel Anti-HIV Drugs. for presentation at the Amer. Soc. of Pharmacol. & Exptl. Therapeutics Meeting in San Francisco, CA July 30, 1993.

16. Mascola, J., **O. Weislow, S. Snyder, S. Belay**, F. McCutchan, J. McNeil, D. Burke and M.C. Walker. Neutralizing Antibody Activity in Sera from Human Immunodeficiency Virus Type-1 Vaccine Recipients from the AIDS Vaccine Clinical Trials Network. Sixth NCVDG Meeting, Conference on Advances in AIDS Vaccine Development, Washington D.C., 1993.

17. Mayers, D.L., Wagner, K.F., Chung, R.C.Y., **Lane, J.R.**, Vahey, M.T., **White, F.A.**, Ruiz, N.M., Hicks, C.B., **Weislow, O.S.**, Gardner, L.I., Burke, D.S. and the RV43 Study Group, Zidovudine (AZT) Resistance is Temporally Associated with Clinical Failure in Patients on AZT Therapy. for presentation at the First National Conf. on Human Retroviruses and Related Infections, Washington, D.C. Dec. 1993.

18. Mayers DL, Wagner KF, Chung RCY, Lane JR, Vahey MT, White FA, Ruiz NM, Hicks CBN, Weislow OS, Gardner LI, and Burke DS. Zidovudine (AZT) resistance is temproally associated with clinical failure in patients on AZT therapy. First National Conference on Human Retroviruses and Related Infections, December 1993, Washington D.C.

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20. Mosca JD, Kaushal S, Larussa V, Kessler S, Gartner S, Kim J, Perera P, Yu Z, Ritchey D, Xu J, Rosenberg Y, St Louis D, Weislow O, Mayers D and Burke D. Human bone marrow-derived CD34+ cells as targets for gene therapy against HIV infection. Second National Conference on Human Retroviruses and Related Infections, December 1994, Washington D.C.

21. **White, F.A., Tran, M., Zhao, X., Antezana, M., Weislow, O.**, Vahey, M. and Mayers, D.L.,

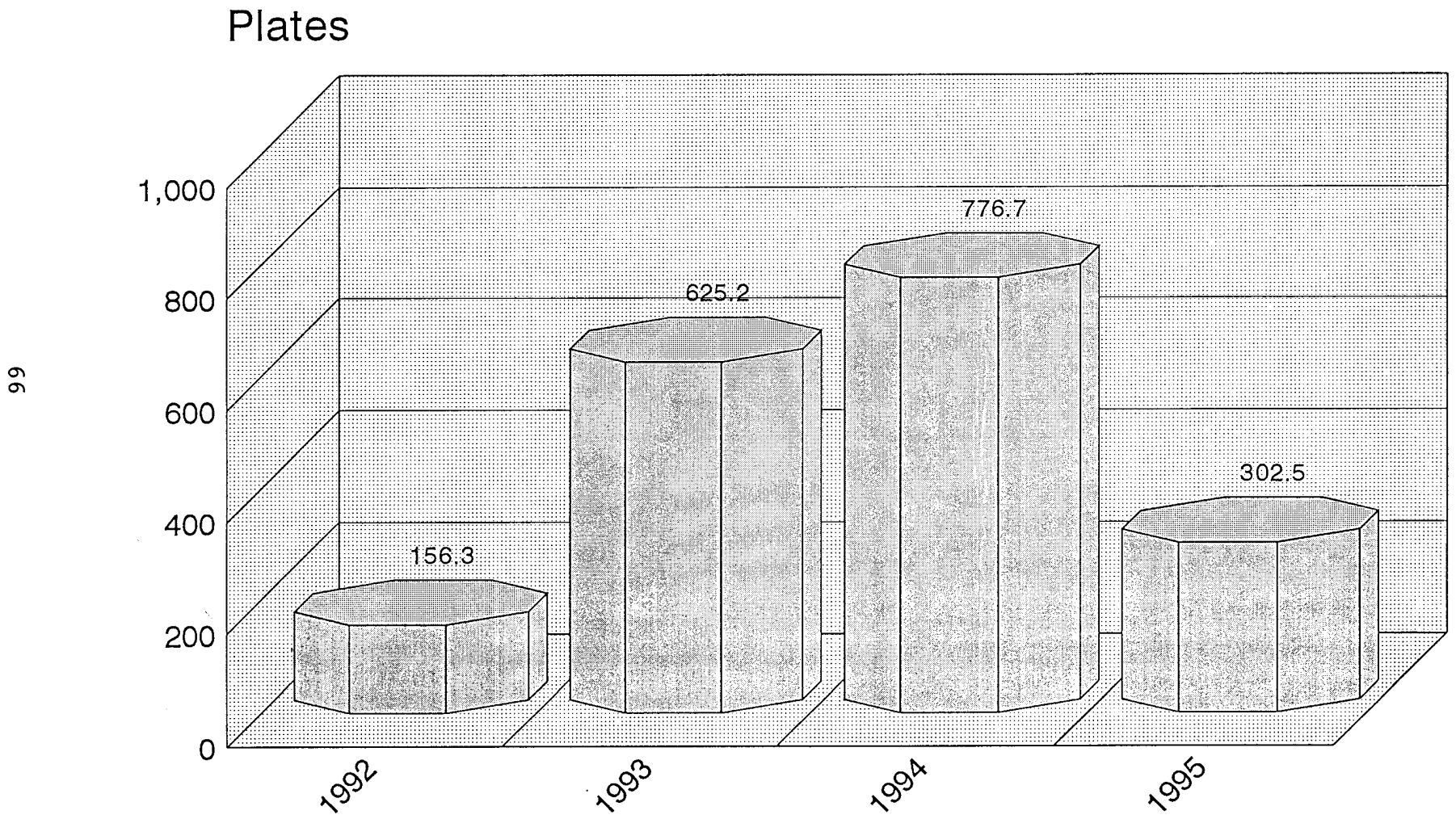
An Improved 215 PCR Assay for DNA and RNA. presented at the Third Workshop on Viral Resistance Gaithersburg Hilton, Sept 19-23, 1993.

22. **Weislow, O.S., S. Belay, S. Snyder, M. Yeager**, F. McCutchan, J. Mascola and D.L. Mayers. Optimization of Peripheral Blood Mononuclear Cell (PBMC)-Based Neutralization Assays for Early Passsage Clinical Isolates of HIV-1. Sixth NCVDG Meeting, Conference on Advances in AIDS Vaccine Development, Washington D.C., 1993.

II Summary of Services Provided

AC Plate Totals

490 CONTRACT OCT92 - SEP95



92 & 93 totals averaged!!

Contract 490 Final Summary
Summary Table

	NUMBERS PERFORMED			
	FY93	FY94	FY95	TOTAL
Specimens Recieved	919	420	10	1349
Cocultures setup	491	263	10	764
Successful Virus Expansions	253	128	18	399
Virus Titrations	10416	5376	0	15792
4-Drug Assays	11286	5130	0	16416
Syncytium Induction Assays	723	1668	54	2445
RNA and DNA 215-Point Mutation Assays	0	1083	0	1083
Specimens Recieved	0	367	1168	1535
Cocultures setup	0	52	163	215
Successful Virus Expansions	0	24	132	156
Virus Titrations	0	1386	4830	6216
4-Drug Assays	0	1881	6555	8436
Syncytium Induction Assays	0	87	327	414
RNA and DNA 215-Point Mutation Assays	0	188	600	788
Specimens Recieved	314	493	490	1297
Cocultures setup	283	463	488	1234
Successful Virus Expansions	106	193	160	459
Virus Titrations	0	294	1176	1470
4-Drug Assays	0	0	1425	1425
RNA and DNA 215-Point Mutation Assays	0	0	0	0
Viral Burden: Roche Amplicor	0	0	250	250
Specimens Recieved	19	22	9	50
Cocultures setup	32	41	14	87
Successful Virus Expansions	29	26	13	68
Virus Titrations	1344	1722	588	3654
4-Drug Assays	0	0	1254	1254
Immunotyping				
Virus Titrations--AVEG	0	294	0	294
Neutralization Screening--AVEG	0	84	0	84
Serum Titration--AVEG	0	870	0	870
Virus Expansions--AVEG	0	7	0	7
Virus Titrations--WHO	0	336	0	336
Neutralization Screening--WHO	0	96	0	96
Serum Titration--WHO	0	420	0	420
Virus Expansions--WHO	0	20	0	20
Virus Titrations--R&D	0	4116	0	4116
Neutralization Screening--R&D	0	30	0	30
Serum Titration--R&D	12	360	0	372
Virus Expansions--R&D	0	98	0	98
Syncytium Induction Assays	0	42	0	42
Evaluation				
Cell Culture Maintaince	0	104	24	128
Mycoplasma Evaluation and Treatment	0	57	12	69
Virus Expansions	12	16	2	30
Antiviral Gene Evaluations	0	104	13	117
Reverse Transcriptase Assays	0	2	2	4

Contract 490 Final Summary
Summary Table

	NUMBERS PERFORMED			
	FY93	FY94	FY95	TOTAL
Virus Expansions	0	0	23	23
4-Drug Assays for Dr. Chaing	0	0	8	8
4-Drug Assays for Lynx	0	0	22	22
4-Drug Assays for Dr. Mellor	0	0	2	2
4-Drug Assays for Dr. Schmidt	0		28	28
215 for seroconverters		143		
RV77, Dr. Robb, Facs, etc		164		
DNA Sequencing		28	13	
Molecular Biology R&D		650		
Support Services				
p24/Tissue cultures	65646	65243	25299	156299
Repository: Vials added	14674	15657	14509	44840